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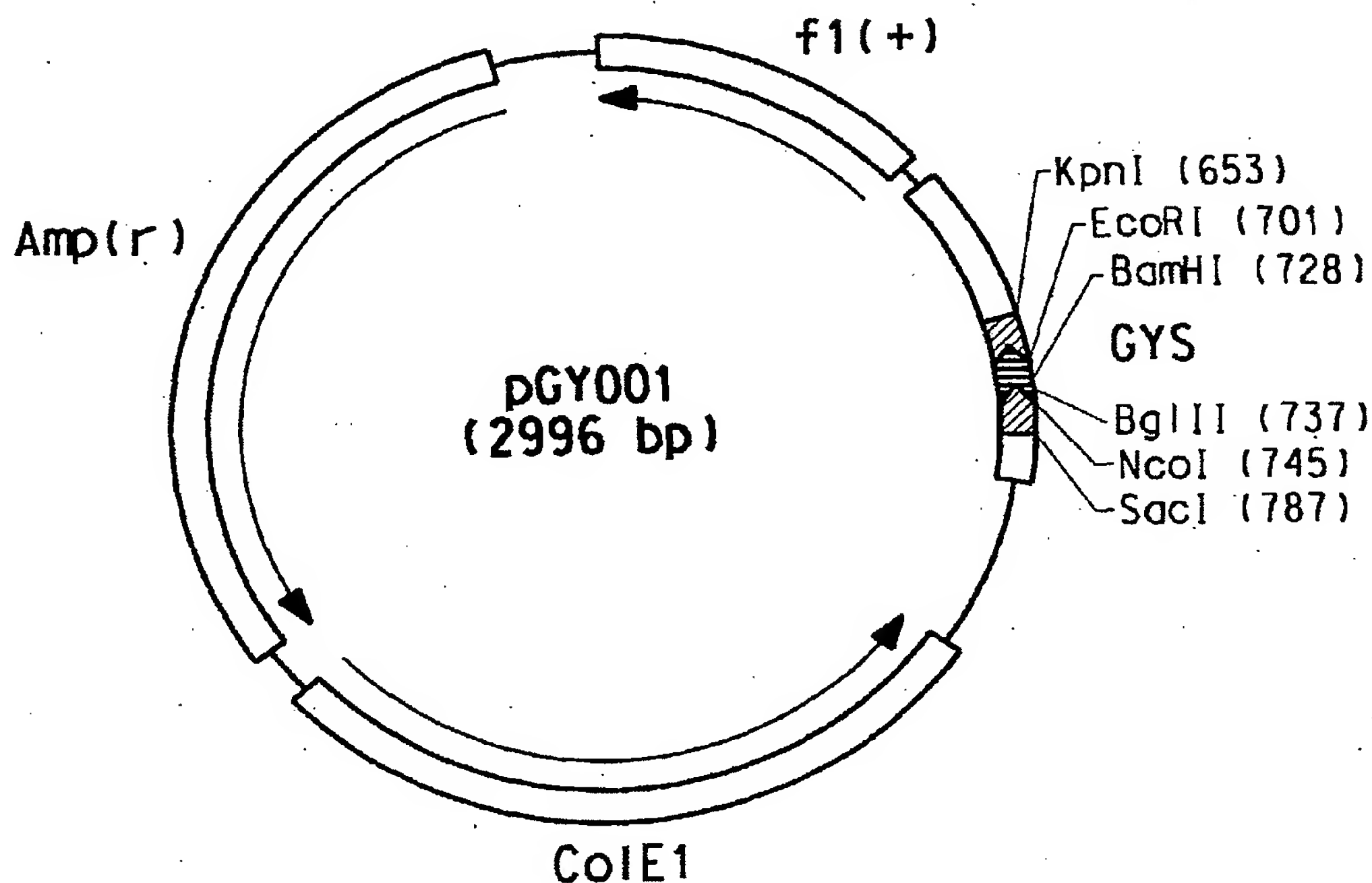
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(54) Title: **PRODUCTION OF SILK-LIKE PROTEINS IN PLANTS**



(57) Abstract: The invention provides methods for the production of silks and silk-like proteins (SLP's) in green plants. Expression of SLP's has been achieved in both seed and leaf tissue in green plants.

**WO 01/90389 A2**

TITLE

## PRODUCTION OF SILK-LIKE PROTEINS IN PLANTS

FIELD OF THE INVENTION

The invention relates to the field of molecular biology and plant genetics.

5 More specifically, this invention describes a technique to produce silk-like proteins plant expression systems.

BACKGROUND OF THE INVENTION

Increasing demands for materials and fabrics that are both light-weight and flexible without compromising strength and durability has created a need for new  
10 fibers possessing higher tolerances for such properties as elasticity, denier, tensile strength and modulus. The search for a better fiber has led to the investigation of fibers produced in nature, some of which possess remarkable qualities. One of those fibers is silk, a group of externally spun fibrous protein secretions.

Silks are produced by over 30,000 species of spiders and by many other  
15 insects particularly in the order *Lepidoptera* (Foelix, R. F. (1992) Biology of Spiders, Cambridge, MA Harvard University Press). Few of these silks have been studied in detail. The cocoon silk of the domesticated silkworm *Bombyx mori* and the dragline silk of the orb-weaving spider *Nephila clavipes* are among the best characterized. Although the structural proteins from the cocoon silk and the  
20 dragline silk are quite different from each other in their primary amino acid sequences, they share remarkable similarities in many aspects. They are extremely glycine and alanine-rich proteins. Fibroin, a structural protein of the cocoon silk, contains 42.9% glycine and 30% alanine. Spidroin 1, a major component of the dragline silk, contains 37.1% glycine and 21.1% alanine. They  
25 are also highly repetitive proteins. The conserved crystalline domains in the heavy chain of the Fibroin and a stretch of polyalanine in Spidroin 1, are repeated numerous times throughout entire molecules. These crystalline domains are surrounded by larger non-repetitive amorphous domains in every 1 to 2 kilobases in the heavy chain of Fibroin, and by shorter repeated GXG amorphous domains  
30 in tandem in Spidroin 1. They are also shear sensitive due to their high copy number of the crystalline domains. During fiber spinning, the crystalline repeats are able to form anti-parallel  $\beta$ -pleated sheets, so that silk protein is turned into semi-crystalline fiber with amorphous flexible chains reinforced by strong and stiff crystals (Kaplan et al., (1997) in Protein-Based Materials, McGrath, K., and  
35 Kaplan, D. Eds, Birkhauser, Boston, pp 104-131).

Traditional silk production from silkworm involves growing mulberry leaves, raising silkworm, harvesting cocoons, and processing of silk fibers. It is labor intensive and time consuming and therefore prohibitively expensive. The

natural defects of the silkworm silk, such as the tendency to wrinkle and the irregularity of fiber diameter further limits its application. Similarly, the mass production of the dragline silk from spiders is not plausible because only small amounts are available from each spider. Furthermore, multiple forms of spider silks are produced simultaneously by any given spider. The resulting mixture has less application than a single isolated silk because the different spider silk proteins have different properties and are not easily separated. Thus, the prospect of producing commercial quantities of spider silk from a natural source is not a practical one and there remains a need for an alternate mode of production.

By using molecular recombination techniques, one can introduce foreign genes or artificially synthesized DNA fragments into different host organisms for the purpose of expressing desired protein products in commercially useful quantities. Such methods usually involve joining appropriate fragments of DNA to a vector molecule, which is then introduced into a recipient organism by transformation. Transformants are selected using a selectable marker on the vector, or by a genetic or biochemical screen to identify the cloned fragment.

While the techniques of foreign gene expression in the host cell are well known in the art and widely practiced, the synthesis of fiber forming foreign polypeptides containing high numbers of repeating units poses unique problems. Genes encoding proteins of this type are prone to genetic instability due to the repeating sequences which result in truncated product instead of the full size protein.

In spite of the above mentioned difficulties, the expression of fiber forming proteins is known in the art. Ferrari et al. (U.S. 5,770,697) disclose methods and compositions for the production of polypeptides having repetitive oligomeric units such as those found in silk-like proteins (SLPs) and elastin-like proteins by the synthetic structural genes. The DNA sequences of Ferrari encode peptides containing an oligopeptide repeating units which contains at least 3 different amino acids and a total of 4-30 amino acids, there being at least 2 repeating units in the peptide and at least 2 identical amino acids in each repeating unit.

The cloning and expression of silk proteins of *B. mori* are also known. Ohshima et al. (*Proc. Natl. Acad. Sci. USA*, 74, 5363 (1977)) reported the cloning of the silk Fibroin gene complete with flanking sequences of *B. mori* into *E. coli*. Petty-Saphon et al. (EP 320702) disclose the recombinant production of silk Fibroin and silk Sericin from a variety of host including *E. coli*, *Saccharomyces cerevisiae*, *Pseudomonas sp.*, *Rhodopseudomonas sp.*, *Bacillus sp.*, and *Streptomyces sp.*

Progress has also been made in the cloning and expression of spider silk proteins. Xu et al. (*Proc. Natl. Acad. Sci. USA*, 87, 7120 (1990)) report the determination of the sequence for a portion of the repetitive sequence of a dragline like protein, Spidroin 1, from the spider *Nephila clavipes*, based on a partial cDNA clone.

Lewis et al. (EP 452925) disclose the expression of spider silk proteins (Spidroin 1 and 2) including protein fragment and variants, of *Nephila clavipes* from transformed *E. coli*.

Lombardi et al. (U.S. 5,245,012) teach the production of recombinant spider silk protein comprising an amorphous domain or subunit a crystalline domain or subunit where the domain or subunit refers to a portion of the protein containing a repeating amino acid sequence that provides a particular mechanostuctural property.

The recent advances in cDNA sequencing of cocoon silk and dragline silk have permitted the synthesis of artificial genes for silk-like proteins (SLPs) with sequence and structural similarity to the native proteins. These artificial genes mimicked sequence arrays of natural cocoon silk from *B. mori* and dragline silk from *N. clavipes*, and had been introduced into microorganisms such as *Escherichia coli*, *Pichia pastoris*, and *Saccharomyces cerevisiae*. SLPs had been produced in these microorganisms through fermentation [Cappello, J., Crissman, J. W. (1990) *Polymer Preprints* 31:193-194; Cappello et al., (1990) *Biotechnol. Prog.* 6:198-202; Fahnestock and Irwin, *Appl. Microbiol. Biotechnol.* (1997), 47(1), 23-32; Prince et al, (1995) *Biochemistry* 34:10879-10885; Fahnestock and Bedzyk, 1997, *Appl. Microbiol. Biotechnol.* (1997), 47(1), 33-39 and commonly owned WO 9429450].

Plants are becoming a favorite host for foreign gene expression. Many recombinant proteins have been produced in transgenic plants (Franken et al., *Curr. Opin. Biotechnol.* 8:411-416, (1997); Whitelam et al., *Biotechnol. Genet. Eng. Rev.* 11:1-29, (1993). Plant genetic engineering combines modern molecular recombination technology and agricultural crop production. Although a variety of silk-like and fiber forming proteins have been expressed in microbial systems, similar expression systems have not been developed in plants. Zhang et al. teach the expression of an elastin-based protein polymer in transgenic tobacco plants (Zhang et al., *Plant Cell Rep.* (1996), 16(3-4), 174-179). Although this represents the expression of a repetitive sequence in plants, the elastin polypeptide bears little resemblance to silk-like peptides and thus the feasibility of SLP expression in plants can not be predicted based on this work.



To date, there are no reported examples of recombinant silk or SLP production in plants. One possible explanation for this lies in the the striking compositional and structural differences between Silks and SLP's and native plant proteins. For example, SLP proteins are very glycine and alanine-rich, highly repetitive, and semi-crystalline in structure. These are characteristics not found in most plant proteins. Thus, introduction and expression of SLP genes in plant cells may pose a number of difficulties. For example, the repetitive sequence of SLP gene may be a target for DNA deletion and rearrangement in plant cells. Alternatively, translation of glycine and alanine-rich SLP might prematurely exhaust glycine and alanine and tRNAs pools in plant cells. Finally, accumulation of semicrystalline SLP may be recognized and degraded by the house-keeping mechanisms in the plant.

The methods recited above for the expression of silk and SLP are useful for production in microbial systems, however fail to teach the production of silk or SLP in plants. The use of a plant platform for the production of silk and silk-like proteins has several advantages over a microbial platform. For example, as a renewable resource, a plant platform requires far less energy and materiel consumption than microbial methods. Similarly, a plant platform represents a far greater available biomass for protein production than a microbial system. Finally, the fact that silks are natural proteins suggests production of high levels of silk will not be toxic to the host.

The problem to be solved, therefore is to provide a method to produce synthetic silk or SLP in commercially useful quantities at relatively low cost. Applicants have solved the stated problem by providing a method to express and produce silk or SLP using plant expression systems.

#### SUMMARY OF THE INVENTION

The present invention provides a method for the production of silk-like proteins in a green plant comprising:

- a) providing a green plant containing a SLP expression cassette having the following structure:

P-SLP-T

wherein:

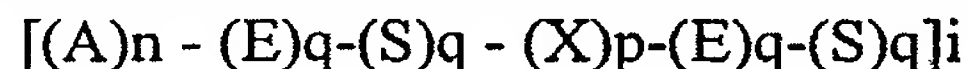
- P is a promoter suitable for driving the expression of a silk-like protein gene;
- SLP is a transgene encoding a mature silk-like protein; and
- T is a 5' terminator;

wherein each of P, SLP and T are operably linked such that expression of the cassette results in translation of the silk-like

protein;

- b) growing said green plant under conditions whereby said transgene is expressed and the silk-like protein is produced; and
- c) optionally recovering said silk-like protein.

5 Additionally the invention provides plants comprising an expression cassette expressing a silk-like protein derived from the silks produced by *Bombyx mori* and *Nephila clavipes*. Specifically the silks and silk-like proteins of the present invention may be natural or variants and will have the general formula:



10 wherein:

A or E are different non-crystalline soft segments of about 10 to 25 amino acids having at least 55% Gly;

S is a semi-crystalline segment of about 6 to 12 amino acids having at least 33% Ala, and 50% Gly;

15 X is a crystalline hard segment of about 6-12 amino acids having at least 33% Ala, and 50% Gly; and

wherein,

$n=2, 4, 8, 16, 32, 64, \text{ or } 128$ ;

$q=0, 1, 2, 4, 8, 16, 32, 64, \text{ or } 128$ ;

20  $p=2, 4, 8, 16, 32, 64, \text{ or } 128$ ;

$i=1-128$ ; and

where  $p \geq n$  or  $q$ .

#### BRIEF DESCRIPTION OF THE DRAWINGS

#### SEQUENCE DESCRIPTIONS AND DEPOSITS

25 Figure 1 is a plasmid map of pGY001 carrying the GYS adapter.

Figure 2A is a plasmid map of pGY101 carrying the DP-1B.8P gene.

Figure 2B is a plasmid map of pGY102 carrying the DP-1B.16P gene.

Figure 3A is a plasmid map of pML63 carrying a 35S/Cab22l promoter driving a GUS reporter.

30 Figure 3B is a plasmid map of pCW109 carrying the  $\beta$ -conglycinin promoter.

Figure 4A is a plasmid map of pGY201 carrying the DP-1B.8P gene under the control of the 35S/Cab22l promoter.

35 Figure 4B is a plasmid map of pGY202 carrying the DP-1B.16P gene under the control of the 35S/Cab22l promoter.

Figure 5A is a plasmid map of pGY211 carrying the DP-1B.8P under the control of the  $\beta$ -conglycinin promoter.

Figure 5B is a plasmid map of pGY213 carrying the DP-1B.8P under the control of the  $\beta$ -conglycinin promoter having a reduced number of restriction sites.

5 Figure 6 is a plasmid map of binary vector pZBL1 carrying a T-DNA region with a NOS promoter driven NPTII gene.

Figure 7A is a plasmid map of pGY401, in which the T-DNA region includes an expression cassette comprising DP-1B.8P under the control of the 35S/Cab221 promoter in conjunction with the NOS driven NPTII.

10 Figure 7B is a plasmid map of pGY402 harboring an expression cassette containing DP-1B.16.P under the control of the 35S/Cab221 promoter within the T-DNA region.

Figure 8A is a plasmid map of pGY411 in which the T-DNA region includes the DP-1B8.P gene under the control of the  $\beta$ -conglycinin promoter.

15 Figure 8B is a plasmid map of pGY412 carrying the DP-1B16.P gene under the control of the  $\beta$ -conglycinin promoter within the T-DNA region.

Figure 9A is an immunoblot showing accumulation of DP-1B protein in leaves and seeds of T1 transgenic Arabidopsis.

Figure 9B is an immunoblot showing complete C-terminus of the DP-1B protein.

20 Figure 9C is a DNA agrose gel showing the transgene in Arabidopsis chromosome.

Figure 10A is an immunoblot showing accumulation of DP-1B protein in leaves and seeds of T2 transgenic Arabidopsis.

25 Figure 10B is a DNA agrose gel showing the transgene in the chromosome of T2 Arabidopsis.

Figure 11A is a plasmid map of pZBL102 carrying the HPT gene under the control of the 35S promoter.

Figure 11B is a plasmid map of pGY220 carrying the DP-1B.16P under the control of the  $\beta$ -conglycinin promoter.

30 Figure 12A is a plasmid map of pLS3 carrying the  $\beta$ -conglycinin promoter – DP-1B.8P construct for transformation of soy embryos.

Figure 12B is a plasmid map of pLS4 carrying the  $\beta$ -conglycinin promoter – DP-1B.16P construct for transformation of soy embryos.

35 Figure 13A is an immunoblot showing accumulation of DP-1B protein in transgenic soy somatic embryos.

Figure 13B is an immunoblot showing complete C-terminus of the DP-1B protein.

Figure 13C is a DNA agrose gel showing the transgene in chromosome of soy somatic embryo.

Figure 14A is a coomassie blue staining of total protein profiles in the purification fractions from *Arabidopsis* plant rosettes used in Example 8.

5 Figure 14B is an immunoblot detection of DP-1B protein in the purification fractions from Figure 14A.

Applicants made the following biological deposits under the terms of the Budapest Treaty on the International Recognition of the Deposit of Micro-organisms for the Purposes of Patent Procedure:

10

Depositor Identification Reference	International Depository Designation	Date of Deposit
pGY401	ATCC PTA-1912	May 24, 2000
pLS3	ATCC PTA-1911	May 24, 2000

Applicant(s) have provided 29 sequences in conformity with 37 C.F.R. 1.821-1.825 ("Requirements for Patent Applications Containing Nucleotide Sequences and/or Amino Acid Sequence Disclosures - the Sequence Rules") and consistent with World Intellectual Property Organization (WIPO) Standard ST.25 (1998) and the sequence listing requirements of the EPO and PCT (Rules 5.2 and 49.5(a-bis), and Section 208 and Annex C of the Administrative Instructions). The symbols and format used for nucleotide and amino acid sequence data comply with the rules set forth in 37 C.F.R. §1.822.

20

Sequence Description	SEQ ID NO: Nucleic acid	SEQ ID NO: Amino acid
Spirdroin 1		1
SLP repeat unit		2
SLP repeat unit		3
Peptide SLP		4
SLP repeat unit		5
SLP repeat unit		6
SLP repeat unit		7
Spider silk variant		8
Spider silk variant repeat unit		9
DP-1A		10
DP-1B		11
Spider silk repeat unit		12
DP-1B 809 amino acid repeat		13



Sequence Description	SEQ ID NO: Nucleic acid	SEQ ID NO: Amino acid
DP-1B 1617 amino acid repeat		14
Primer	15	
Primer	16	
Peptide adapter		17
Sense DNA strand encoding a peptide adapter	18	
Anti-sense DNA strand encoding a peptide adapter	19	
Adapter peptide		20
Gene encoding DP-1B 8-mer	21	
DP-1B 8mer		22
Gene encoding DP-1B 16-mer	23	
DP-1B 16-mer		24
Spider silk repeat unit		25
Primer	26	
Primer	27	
Primer	28	
Primer	29	

### DETAILED DESCRIPTION OF THE INVENTION

The present invention provides methods for of the production of silks and  
 5 silk-like proteins in green plants. The methods allow for the more cost effective  
 production of silk heretofore not obtainable from natural or microbial sources.  
 The silks and silk-like proteins of the present invention may have properties  
 suitable for fabrics, or alternatively may be useful in materials construction. For  
 example the spider dragline silk has a tensile strength of over 200 ksi with an  
 10 elasticity of nearly 35%, which makes it more difficult to break than either  
 KEVLAR® fibers or steel. When spun into fibers, spider silk may have  
 application in the bulk clothing industries as well as being applicable for certain  
 kinds of high strength uses such as rope, surgical sutures, flexible tie downs for  
 certain electrical components and even as a biomaterial for implantation (e.g.,  
 15 artificial ligaments or aortic banding). Additionally these fibers may be mixed  
 with various plastics and/or resins to prepare a fiber-reinforced plastic and/or resin  
 product.

In this disclosure, a number of terms and abbreviations are used. The  
 following definitions are provided.

20 "Open reading frame" is abbreviated ORF.

“Polymerase chain reaction” is abbreviated PCR.

The term “silk-like protein” will be abbreviated SLP and refers to natural silk proteins and their synthetic analogs having the following three criteria:

- 5 (1) Amino acid composition of the molecule is dominated by glycine and/or alanine; (2) Consensus crystalline domain is arrayed repeatedly throughout the molecule; (3) The molecule is shear sensitive and can be spun into semicrystalline fiber. SLP's should also include molecules which are the modified variants of the natural silk proteins and their synthetic analogs defined above.

10 The terms “peptide”, “polypeptide” and “protein” are used interchangeably.

The term “spider silk variant protein” will refer to a designed protein, the amino acid sequence of which is based on repetitive sequence motifs and variations thereof that are found in a known a natural spider silk.

15 The term “DP-1B” will refer to any spider silk variant derived from the amino acid sequence of the natural Protein 1 (Spidroin 1) of *Nephila calvipes* as set forth in SEQ ID NO:1.

20 As used herein, an “isolated nucleic acid fragment” is a polymer of RNA or DNA that is single- or double-stranded, optionally containing synthetic, non-natural or altered nucleotide bases. An isolated nucleic acid fragment in the form of a polymer of DNA may be comprised of one or more segments of cDNA, genomic DNA or synthetic DNA.

25 “Gene” refers to a nucleic acid fragment that expresses a specific protein, including regulatory sequences preceding (5' non-coding sequences) and following (3' non-coding sequences) the coding sequence. “Native gene” refers to a gene as found in nature with its own regulatory sequences. “Chimeric gene” refers any gene that is not a native gene, comprising regulatory and coding sequences that are not found together in nature. Accordingly, a chimeric gene may comprise regulatory sequences and coding sequences that are derived from different sources, or regulatory sequences and coding sequences derived from the same source, but arranged in a manner different than that found in nature.

30 “Endogenous gene” refers to a native gene in its natural location in the genome of an organism. A “foreign” gene or “transgene” refers to a gene not normally found in the host organism, but that is introduced into the host organism by gene transfer. Foreign genes can comprise native genes inserted into a non-native organism, or chimeric genes. A “transgene” is a gene that has been introduced into the genome by a transformation procedure.

35 “Synthetic genes” can be assembled from oligonucleotide building blocks that are chemically synthesized using procedures known to those skilled in the art.

These building blocks are ligated and annealed to form gene segments which are then enzymatically assembled to construct the entire gene. "Chemically synthesized", as related to a sequence of DNA, means that the component nucleotides were assembled *in vitro*. Manual chemical synthesis of DNA may be accomplished using well established procedures, or automated chemical synthesis can be performed using one of a number of commercially available machines. Accordingly, the genes can be tailored for optimal gene expression based on optimization of nucleotide sequence to reflect the codon bias of the host cell. The skilled artisan appreciates the likelihood of successful gene expression if codon usage is biased towards those codons favored by the host. Determination of preferred codons can be based on a survey of genes derived from the host cell where sequence information is available.

"Coding sequence" refers to a DNA sequence that codes for a specific amino acid sequence. "Suitable regulatory sequences" refer to nucleotide sequences located upstream (5' non-coding sequences), within, or downstream (3' non-coding sequences) of a coding sequence, and which influence the transcription, RNA processing or stability, or translation of the associated coding sequence. Regulatory sequences may include promoters, translation leader sequences, introns, and polyadenylation recognition sequences.

"Promoter" refers to a DNA sequence capable of controlling the expression of a coding sequence or functional RNA. In general, a coding sequence is located 3' to a promoter sequence. Promoters may be derived in their entirety from a native gene, or be composed of different elements derived from different promoters found in nature, or even comprise synthetic DNA segments. It is understood by those skilled in the art that different promoters may direct the expression of a gene in different tissues or cell types, or at different stages of development, or in response to different environmental conditions. Promoters which cause a gene to be expressed in most cell types at most times are commonly referred to as "constitutive promoters". It is further recognized that since in most cases the exact boundaries of regulatory sequences have not been completely defined, DNA fragments of different lengths may have identical promoter activity.

"Regulated promoter" refers to promoters that direct gene expression not constitutively but in a temporally- and/or spatially-regulated manner and include both tissue-specific and inducible promoters. It includes natural and synthetic sequences as well as sequences which may be a combination of synthetic and natural sequences. Different promoters may direct the expression of a gene in different tissues or cell types, or at different stages of development, or in response to different environmental conditions. New promoters of various types useful in

plant cells are constantly being discovered; numerous examples may be found in the compilation by Okamuro et al., *Biochemistry of Plants* 15:1-82, 1989. Since in most cases the exact boundaries of regulatory sequences have not been completely defined, DNA fragments of different lengths may have identical promoter activity.

"Tissue-specific promoter" refers to regulated promoters that are not expressed in all plant cells but only in one or more cell types in specific organs (such as leaves or seeds), specific tissues (such as embryo or cotyledon), or specific cell types (such as leaf parenchyma or seed storage cells). These also include promoters that are temporally regulated, such as in early or late embryogenesis, during fruit ripening in developing seeds or fruit, in fully differentiated leaf, or at the onset of senescence.

The term "complementary" is used to describe the relationship between nucleotide bases that are capable to hybridizing to one another. For example, with respect to DNA, adenosine is complementary to thymine and cytosine is complementary to guanine.

The "3' non-coding sequences" refer to DNA sequences located downstream of a coding sequence and include polyadenylation recognition sequences and other sequences encoding regulatory signals capable of affecting mRNA processing or gene expression. The polyadenylation signal is usually characterized by affecting the addition of polyadenylic acid tracts to the 3' end of the mRNA precursor.

The term "operably linked" refers to the association of nucleic acid sequences on a single nucleic acid fragment so that the function of one is affected by the other. For example, a promoter is operably linked with a coding sequence when it is capable of affecting the expression of that coding sequence (i.e., that the coding sequence is under the transcriptional control of the promoter). Coding sequences can be operably linked to regulatory sequences in sense or antisense orientation.

The term "expression", as used herein, refers to the transcription and stable accumulation of sense (mRNA) or antisense RNA derived from the nucleic acid fragment of the invention. Expression may also refer to translation of mRNA into a polypeptide.

"Mature" protein refers to a post-translationally processed polypeptide; i.e., one from which any pre- or propeptides present in the primary translation product have been removed.

"Transformation" refers to the transfer of a nucleic acid fragment into the genome of a host organism, resulting in genetically stable inheritance. Host



organisms containing the transformed nucleic acid fragments are referred to as “transgenic” or “recombinant” or “transformed” organisms.

The terms “plasmid”, “vector” and “cassette” refer to an extra chromosomal element often carrying genes which are not part of the central metabolism of the cell, and usually in the form of circular double-stranded DNA molecules. Such elements may be autonomously replicating sequences, genome integrating sequences, phage or nucleotide sequences, linear or circular, of a single- or double-stranded DNA or RNA, derived from any source, in which a number of nucleotide sequences have been joined or recombined into a unique construction which is capable of introducing a promoter fragment and DNA sequence for a selected gene product along with appropriate 3' untranslated sequence into a cell. “Transformation cassette” refers to a specific vector containing a foreign gene and having elements in addition to the foreign gene that facilitate transformation of a particular host cell. “Expression cassette” refers to a specific vector containing a foreign gene and having elements in addition to the foreign gene that allow for enhanced expression of that gene in a foreign host.

As used herein the following abbreviations will be used to identify specific amino acids:

Amino Acid	Three-Letter Abbreviation	One-Letter Abbreviation
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Asparagine or aspartic acid	Asx	B
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamine acid	Glu	E
Glutamine or glutamic acid	Glx	Z
Glycine	Gly	G
Histidine	His	H
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P

Amino Acid	Three-Letter Abbreviation	One-Letter Abbreviation
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

Standard recombinant DNA and molecular cloning techniques used here are well known in the art and are described by Sambrook, J., Fritsch, E. F. and  
 5 Maniatis, T., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1989) (hereinafter "Maniatis"); and by Silhavy, T. J., Bennis, M. L. and Enquist, L. W., Experiments with Gene Fusions, Cold Spring Harbor Laboratory Cold Press Spring Harbor, NY (1984); and by Ausubel, F. M. et al., Current Protocols in  
 10 Molecular Biology, published by Greene Publishing Assoc. and Wiley-Interscience (1987).

#### Expression Cassette

The present invention provides a method for the production of silk-like proteins in plants. The method proceeds by providing a plant expression cassette  
 15 having a DNA construct comprising a promoter, a transgene encoding a silk-like protein and a 5' terminator region. Expression of the transgene may be constitutive or regulated.

Promoters useful for driving the expression of foreign genes in plant hosts are common and well known in the art. It may be useful to have the present SLP  
 20 transgene expressed constitutively or in a regulated fashion. Constitutive plant promoters are well known. Some suitable promoters include but are not limited to the nopaline synthase promoter, the octopine synthase promoter, CaMV 35S promoter, the ribulose-1,5-bisphosphate carboxylase promoter, Adh1-based pEmu, Act1, the SAM synthase promoter and Ubi promoters and the promoter of  
 25 the chlorophyll a/b binding protein.

Alternatively it may be desired to have the SLP transgene expressed in a regulated fashion. Regulated expression of the SLP's is possible by placing the coding sequence of the silk-like protein under the control of promoters that are tissue-specific, developmental-specific, or inducible.

30 Several tissue-specific regulated genes and/or promoters have been reported in plants. These include genes encoding the seed storage proteins (such

as napin, cruciferin, .beta.-conglycinin, glycinin and phaseolin), zein or oil body proteins (such as oleosin), or genes involved in fatty acid biosynthesis (including acyl carrier protein, stearoyl-ACP desaturase, and fatty acid desaturases (fad 2-1)), and other genes expressed during embryo development (such as Bce4, see, for  
5 example, EP 255378 and Kridl et al., *Seed Science Research* (1991) 1:209-219). Particularly useful for seed-specific expression is the pea vicilin promoter [Czako et al., *Mol. Gen. Genet.* (1992), 235(1), 33-40]. Other useful promoters for expression in mature leaves are those that are switched on at the onset of senescence, such as the SAG promoter from *Arabidopsis* [Gan et al., Inhibition of  
10 leaf senescence by autoregulated production of cytokinin, *Science* (Washington, DC) (1995), 270 (5244), 1986-8].

A class of fruit-specific promoters expressed at or during anthesis through fruit development, at least until the beginning of ripening, is discussed in U.S. 4,943,674, the disclosure of which is hereby incorporated by reference.  
15 cDNA clones that are preferentially expressed in cotton fiber have been isolated [John et al., Gene expression in cotton (*Gossypium hirsutum* L.) fiber: cloning of the mRNAs, *Proc. Natl. Acad. Sci. U.S.A.* (1992), 89 (13), 5769-73]. cDNA clones from tomato displaying differential expression during fruit development have been isolated and characterized [Mansson et al., *Mol. Gen. Genet.* (1985)  
20 200:356-361; Slater et al., *Plant Mol. Biol.* (1985) 5:137-147]. The promoter for polygalacturonase gene is active in fruit ripening. The polygalacturonase gene is described in U.S. Patent No. 4,535,060 (issued August 13, 1985), U.S. Patent No. 4,769,061 (issued September 6, 1988), U.S. Patent No. 4,801,590 (issued January 31, 1989) and U.S. Patent No. 5,107,065 (issued April 21, 1992), which  
25 disclosures are incorporated herein by reference.

Mature plastid mRNA for psbA (one of the components of photosystem II) reaches its highest level late in fruit development, in contrast to plastid MRNAs for other components of photosystem I and II which decline to nondetectable levels in chromoplasts after the onset of ripening [Piechulla et al., *Plant Mol. Biol.*  
30 (1986) 7:367-376]. Recently, cDNA clones representing genes apparently involved in tomato pollen [McCormick et al., *Tomato Biotechnology* (1987) Alan R. Liss, Inc., New York) and pistil (Gasser et al., *Plant Cell* (1989), 1:15-24] interactions have also been isolated and characterized.

Other examples of tissue-specific promoters include those that direct  
35 expression in leaf cells following damage to the leaf (for example, from chewing insects), in tubers (for example, patatin gene promoter), and in fiber cells (an example of a developmentally-regulated fiber cell protein is E6 [John et al., Gene expression in cotton (*Gossypium hirsutum* L.) fiber: cloning of the mRNAs, *Proc.*

*Natl. Acad. Sci. U.S.A.* (1992), 89(13), 5769-73]). The E6 gene is most active in fiber, although low levels of transcripts are found in leaf, ovule and flower.

The termination region used in the expression cassette will be chosen primarily for convenience, since the termination regions appear to be relatively interchangeable. The termination region which is used may be native with the transcriptional initiation region, may be native with the DNA sequence of interest, or may be derived from another source. The termination region may be naturally occurring, or wholly or partially synthetic. Convenient termination regions are available from the Ti-plasmid of *A. tumefaciens*, such as the octopine synthase and nopaline synthase termination regions or from the genes for  $\beta$ -phaseolin, the chemically inducible lant gene, pIN (Hershey et al., Isolation and characterization of cDNA clones for RNA species induced by substituted benzenesulfonamides in corn. *Plant Mol. Biol.* (1991), 17(4), 679-90; U.S. Patent No. 5,364,780).

The transgene encoding the silk or SLP protein may be naturally occurring or may be synthetic. The present transgenes will generally be derived from silk producing organisms such as insects in the order *Lepidoptera* including *Bombyx mori* and *Nephila clavipes*. Genes encoding the subject polypeptides will generally be at least about 900 nucleotides in length, usually at least 1200 nucleotides in length, preferably at least 1500 nucleotides in length. The genes of the subject invention generally comprise concatenated monomers of DNA encoding the same amino acid sequence, where only one repeating unit is present to form a homopolymer, where all or a part of two or more different monomers encoding different amino acid repeating units may be joined together to form a new monomer encoding a block or random copolymer. The individual amino acid repeating units will have from 3 to 20 amino acids (9 to 60 nucleotides), generally 3 to 15 amino acids (9 to 45 nucleotides), usually 3 to 12 amino acids (9 to 36 nucleotides), more usually 3 to 9 amino acids (9 to 27 nucleotides) amino acids, usually having the same amino acid appear at least twice in the same unit, generally separated by at least one amino acid. In some instances, the minimum number of amino acids will be 4. Within a monomer, dsDNA encoding the same amino acid repeating unit may involve two or more nucleotide sequences, relying on the codon redundancy to achieve the same amino acid sequence.

The genes of the subject invention comprise regions comprising repeats of the repetitive units, usually a block of at least 2 units, and up to the entire region of repetitive units. Blocks of repetitive units may be interspersed with individual or blocks of other repetitive units, or intervening sequences. The repeating units may have the same sequence or there may be 2 or more different sequences



employed to encode the repeating unit, using the codon redundancy for a particular amino acid to vary the sequence.

A silk-like-protein (SLP) gene may be produced by providing oligomers or multimers of from about 5 to 25 repeat units as described above, more usually of about 6 to 15 repeat units. By having different cohesive ends, the oligomers may be concatemerized to provide for the polymer having 2 or more of the oligomeric units, usually not more than about 50 oligomeric units, more usually not more than about 30 oligomeric units, and frequently not more than about 25 oligomeric units.

#### 10 Silk and SLP Polypeptides

The present invention provides various silk and silk-like proteins for expression from a plant platform. Of particular interest are polypeptides which have as a repeating unit SGAGAG (SEQ ID NO:2) and GAGAGS (SEQ ID NO:3). This repeating unit is found in a naturally occurring silk fibroin protein, which can be represented as GAGAG(SGAGAG)<sub>8</sub> SGAAGY (SEQ ID NO:4). Particularly suitable in the present invention are silk-like protein having the general formula:

$$[(A)_n - (E)_q - (S)_q - (X)_p - (E)_q - (S)_q]_i$$

wherein:

- 20 A or E are different non-crystalline soft segments of about 10 to 25 amino acids having at least 55% Gly;  
S is a semi-crystalline segment of about 6 to 12 amino acids having at least 33% Ala, and 50% Gly;  
X is a crystalline hard segment of about 6-12 amino acids having at least 33% Ala, and 50% Gly; and

25 wherein,

- n=2, 4, 8, 16, 32, 64, 128;  
q=0, 1, 2, 4, 8, 16, 32, 64, 128;  
p=2, 4, 8, 16, 32, 64, 128;  
30 i=1-128; and

where  $p \geq n$  or  $q$ .

Preferred combinations of the non-crystalline, semi-crystalline or hard segments will include, but are not limited to [(A)<sub>4</sub>-(X)<sub>8</sub>]<sub>8</sub>, [(A)<sub>4</sub>-(X)<sub>8</sub>-(S)]<sub>8</sub>, [(A)<sub>4</sub>-(X)<sub>8</sub>-(E)]<sub>8</sub>, [(A)<sub>8</sub>-(X)<sub>8</sub>]<sub>8</sub>, [(A)<sub>4</sub>-(S)-(X)<sub>8</sub>]<sub>8</sub>, [(A)<sub>4</sub>-(S)<sub>2</sub>-(X)<sub>8</sub>]<sub>8</sub>, [(A)<sub>4</sub>-(E)-(X)<sub>8</sub>-(E)]<sub>8</sub>, [(A)<sub>4</sub>-(E)-(X)<sub>8</sub>]<sub>8</sub>, [(A)<sub>4</sub>-(S)-(X)<sub>8</sub>-(E)]<sub>8</sub>, and [(A)<sub>4</sub>-(S)<sub>2</sub>-(X)<sub>8</sub>-(E)]<sub>8</sub>. Most preferred combinations are these in which the non-crystalline, semi-crystalline or hard segments are defined as follows:

A=SGGAGGAGG (SEQ ID NO:5), E=GPGQQGPGGY (SEQ ID NO:6),  
S=GAGAGY (SEQ ID NO:7), and X=SGAGAG (SEQ ID NO:2).

In a preferred embodiment the silk or SLP may be derived from spider silk. There are a variety of spider silks which may be suitable for expression in plants. Many of these are derived from the orb-weaving spiders such as those belonging to the genus *Nephila*. Silks from these spiders may be divided into major ampullate, minor ampullate, and flagelliform silks, each having different physical properties. For a review of suitable spider silks see Hayashi et al., *Int. J. Biol. Macromol.* (1999), 24(2,3), 271-275, for example. Those of the major ampullate are the most completely characterized and are often referred to as spider dragline silk. Natural spider dragline consists of two different proteins that are co-spun from the spider's major ampullate gland. The amino acid sequence of both dragline proteins has been disclosed by Xu et al., *Proc. Natl. Acad. Sci. U.S.A.*, 87, 7120, (1990) and Hinman and Lewis, *J. Biol. Chem.* 267, 19320 (1992), and will be identified hereinafter as Dragline Protein 1 (DP-1) and Dragline Protein 2 (DP-2). Within the context of the present invention Dragline Protein 1 (DP-1) and Dragline Protein 2 (DP-2) were the focus for spider silk variant design.

The design of the spider silk variant proteins was based on consensus amino acid sequences derived from the fiber forming regions of the natural spider silk dragline proteins of *Nephila clavipes*. The amino acid sequence of a fragment of DP-1 is repetitive and rich in glycine and alanine, but is otherwise unlike any previously known amino acid sequence. The "consensus" sequence of a single repeat, viewed in this way, is:

A GQG GYG GLG XQG A GRG GLG GQG A GAAAAAAGG (SEQ ID NO:8)

where X may be S, G, or N.

Individual repeats differ from the consensus according to a pattern which can be generalized as follows: (1) The poly-alanine sequence varies in length from zero to seven residues. (2) When the entire poly-alanine sequence is deleted, so also is the surrounding sequence encompassing AGRGGLGGQGAG<sub>n</sub>GG (SEQ ID NO:9). (3) Aside from the poly-alanine sequence, deletions generally encompass integral multiples of three consecutive residues. (4) Deletion of GYG is generally accompanied by deletion of GRG in the same repeat. (5) A repeat in which the entire poly-alanine sequence is deleted is generally preceded by a repeat containing six alanine residues.

Synthetic analogs of DP-1 were designed to mimic both the repeating consensus sequence of the natural protein and the pattern of variation among

individual repeats. Two analogs of DP-1 were designed and designated DP-1A and DP-1B. DP-1A is composed of a tandemly repeated 101-amino acid sequence listed in SEQ ID NO10. The 101-amino acid "monomer" comprises four repeats which differ according to the pattern (1)-(5) above. This 101-amino acid long peptide monomer is repeated from 1 to 16 times in a series of analog proteins. DP-1B was designed by reordering the four repeats within the monomer of DP-1A. This monomer sequence, shown in SEQ ID NO:11, exhibits all of the regularities of (1)-(5) above. In addition, it exhibits a regularity of the natural sequence which is not shared by DP-1A, namely that a repeat in which both GYG and GRG are deleted is generally preceded by a repeat lacking the entire poly-alanine sequence, with one intervening repeat. The sequence of DP-1B matches the natural sequence more closely over a more extended segment than does DP-1A.

Thus it is an object of the present invention to provide a spider dragline variant protein wherein the full length variant protein is defined by the formula:  
 $[ACGQGGYGGLGXQGAGRGGGLGGQGAGAnGG]_z$  (SEQ ID NO:12)  
 wherein X=S, G or N; n=0-7 and z=1-75, and wherein the value of z determines the number of repeats in the variant protein and wherein the formula encompasses variations selected from the group consisting of:

- (a) when n=0 the sequence encompassing AGRGGLGGQGAGAnGG (SEQ ID NO:9) is deleted;
  - (b) deletions other than the poly-alanine sequence, limited by the value of n will encompass integral multiples of three consecutive residues;
  - (c) the deletion of GYG in any repeat is accompanied by deletion of GRG in the same repeat; and
  - (d) where a first repeat where n=0 is deleted, the first repeat is preceded by a second repeat where n=6; and
- wherein the full-length protein is encoded by a gene or genes and wherein said gene or genes are not endogenous to the *Nephila clavipes* genome.

The silk variants and SLP's of the present invention will have physical properties commonly associated with natural proteins. So for example, the silks and SLP's will be expected to have tenacities (g/denier) of about 2.8 to about 5.2, tensile strengths (psi) of about 45,000 to about 83,000 and elongations (%) of about 13 to about 31.

### Plant Hosts

Virtually any plant capable of supporting the expression of a silk or SLP gene is suitable as a host in the present invention. Suitable plants will be either monocots or dicots and will preferably be of the sort that are hardy and permit

several harvests per year. Suitable green plants will included but are not limited to soybean, rapeseed, sunflower, cotton, corn, tobacco, alfalfa, wheat, barley, oats, sorghum, rice, *Arabidopsis*, sugar beet, sugar cane, canola, millet, beans, peas, rye, flax, grasses, and banana.

5 A variety of techniques are available and known to those skilled in the art for introduction of constructs into a plant cell host. These techniques include transformation with DNA employing *A. tumefaciens* or *A. rhizogenes* as the transforming agent, electroporation, particle acceleration, etc. [See for example, EP 295959 and EP 138341]. It is particularly preferred to use the binary type  
10 vectors of Ti and Ri plasmids of *Agrobacterium spp.* Ti-derived vectors transform a wide variety of higher plants, including monocotyledonous and dicotyledonous plants, such as soybean, cotton, rape, tobacco, and rice [Pacciotti et al. (1985) *Bio/Technology* 3:241; Byrne et al. (1987) *Plant Cell, Tissue and Organ Culture* 8:3; Sukhapinda et al. (1987) *Plant Mol. Biol.* 8:209-216; Lorz et al. (1985) *Mol.*  
15 *Gen. Genet.* 199:178; Potrykus (1985) *Mol. Gen. Genet.* 199:183; Park et al., *J. Plant Biol.* (1995), 38(4), 365-71; Hiei et al., *Plant J.* (1994), 6:271-282]. The use of T-DNA to transform plant cells has received extensive study and is amply described [EP 120516; Hoekema, In: The Binary Plant Vector System, Offset-  
20 *drukkerij Kanters B.V.*; Alblasterdam (1985), Chapter V, Knauf, et al., *Genetic Analysis of Host Range Expression by Agrobacterium* In: Molecular Genetics of the Bacteria-Plant Interaction, Puhler, A. ed., Springer-Verlag, New York, 1983, p. 245; and An, et al., *EMBO J.* (1985) 4:277-284]. For introduction into plants, the chimeric genes of the invention can be inserted into binary vectors as described in the examples.

25 Other transformation methods are available to those skilled in the art, such as direct uptake of foreign DNA constructs [see EP 295959], techniques of electroporation [see Fromm et al. (1986) *Nature* (London) 319:791] or high-velocity ballistic bombardment with metal particles coated with the nucleic acid constructs [see Kline et al. (1987) *Nature* (London) 327:70, and see U.S. Patent  
30 No. 4,945,050]. Once transformed, the cells can be regenerated by those skilled in the art. Of particular relevance are the recently described methods to transform foreign genes into commercially important crops, such as rapeseed [see De Block et al. (1989) *Plant Physiol.* 91:694-701], sunflower [Everett et al. (1987) *Bio/Technology* 5:1201], soybean [McCabe et al. (1988) *Bio/Technology* 6:923;  
35 Hinchee et al. (1988) *Bio/Technology* 6:915; Chee et al. (1989) *Plant Physiol.* 91:1212-1218; Christou et al. (1989) *Proc. Natl. Acad. Sci USA* 86:7500-7504; EP 301749], rice [Hiei et al., *Plant J.* (1994), 6:271-282], and corn



[Gordon-Kamm et al. (1990) *Plant Cell* 2:603-618; Fromm et al. (1990) *Biotechnology* 8:833-839].

Transgenic plant cells are then placed in an appropriate selective medium for selection of transgenic cells which are then grown to callus. Shoots are grown from callus and plantlets generated from the shoot by growing in rooting medium. The various constructs normally will be joined to a marker for selection in plant cells. Conveniently, the marker may be resistance to a biocide (particularly an antibiotic such as kanamycin, G418, bleomycin, hygromycin, chloramphenicol, herbicide, or the like). The particular marker used will allow for selection of transformed cells as compared to cells lacking the DNA which has been introduced. Components of DNA constructs including transcription cassettes of this invention may be prepared from sequences which are native (endogenous) or foreign (exogenous) to the host. By "foreign" it is meant that the sequence is not found in the wild-type host into which the construct is introduced. Heterologous constructs will contain at least one region which is not native to the gene from which the transcription-initiation-region is derived.

To confirm the presence of the transgenes in transgenic cells and plants, a polymerase chain reaction (PCR) amplification or Southern blot analysis can be performed using methods known to those skilled in the art. Expression products of the transgenes can be detected in any of a variety of ways, depending upon the nature of the product, and include Western blot and enzyme assay. One particularly useful way to quantitate protein expression and to detect replication in different plant tissues is to use a reporter gene, such as GUS. Once transgenic plants have been obtained, they may be grown to produce plant tissues or parts having the desired phenotype. The plant tissue or plant parts, may be harvested, and/or the seed collected. The seed may serve as a source for growing additional plants with tissues or parts having the desired characteristics.

#### Recovery Methods

The SLP's of the present invention may be extracted and purified from the plant tissue by a variety of methods. Preferred in the present invention is a method involving removal of native plant proteins from homogenized plant tissue by lowering pH and heating, followed by ammonium sulfate fractionation. Briefly, total soluble proteins are extracted from the transgenic plants by homogenizing plant tissues such as seeds and leaves. Native plant proteins are removed by precipitation at pH 4.7 and then at 60°C. The resulting supernatant is then fractionated with ammonium sulfate at 40% saturation. The resulting protein will be on the order of 95% pure. Additional purification may be achieved with conventional gel or affinity chromatography.

Description of the Preferred Embodiments:

In this invention, plants are utilized as a production platform for the production of SLPs. Dragline silk-based SLPs are of particular interest because (1) the structural features of dragline silk represent those of SLPs in general so that its expression should reflect the fate of other similar SLP genes in plants, and (2) the fibers of dragline silk possesses many excellent properties which fit well with criteria of the next generation of fibers.

The present invention was demonstrated in two plant systems, *Arabidopsis* and soy embryo tissue culture. Genes encoding either 8mer or 16mers of a DP-1B spider dragline variant were engineered into an expression cassette under the control of either a 35S constitutive promoter or a  $\beta$ -Conglycine seed specific promoter and having a NOS terminator region. The cassette was transformed into *Agrobacterium*, which was then used to infect *Arabidopsis*. The presence of both the 8mer and 18mer spider silk was confirmed immunologically. Protein determination indicated average expression levels at 0.34% of total soluble protein (approximately 0.07% of dry weight) for the 8mer in leaf tissue and at 0.03% of total soluble protein (approximately 0.006% of dry weight) for the 16mer in leaf tissue. Similarly the 8mer was expressed at an average levels of 1.2% of total protein (approximately 0.24% of dry weight) in seeds and the 16mer was expressed at an average level of 0.78% of total protein (approximately 0.16% of dry weight) in seeds.

The same 8mer and 16mer constructs were used for the transformation of soy embryo tissue culture. SLP expression in soybean is extremely attractive since soybean is one of the major crops globally and it itself is a higher efficient and low cost protein synthesis machine. Because gene expression in soy somatic embryos is equivalent to in soybean seeds, the expression of the SLP genes in the embryos demonstrated the feasibility that SLP can be produced in the transgenic soybean seeds. Transformation was effected by ballistic bombardment. Average expression level of 8-mer SLP in the soy embryo system was 1.0% of total soluble protein (approximately 0.4% of dry weight).

Industrial-scale SLP production from transgenic plants requires a purification scheme mostly based on simple methods such as precipitation, filtration, and centrifugation. Due to their special structure and amino acid composition, DP-1B proteins are very stable in water solution; thus they may be possible to be purified from other plant proteins by utilizing simple methods discussed above. Toward this goal, a pGY401 transgenic *Arabidopsis* plant expressing higher level of DP-1B.8P protein was used in developing the purification scheme. To obtain a large amount of starting material, homozygous

transgenic plant was selected for direct soil growth. T4 homozygous seeds were germinated and grown. The plants were harvested and total protein was fractionated. Each fraction was checked for the presence of DP-1B protein. The majority of DP-1B protein was found to be in  $(\text{NH}_4)_2\text{SO}_4$  precipitation fraction.

- 5 This simple method can remove approximately 95% of plant proteins while concentrating DP-1B protein.

### EXAMPLES

The present invention is further defined in the following Examples. It should be understood that these Examples, while indicating preferred  
10 embodiments of the invention, are given by way of illustration only. From the above discussion and these Examples, one skilled in the art can ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various usages and conditions.

### 15 GENERAL METHODS

Standard recombinant DNA and molecular cloning techniques used in the Examples are well known in the art and are described by Sambrook, J., Fritsch, E. F. and Maniatis, T. Molecular Cloning: A Laboratory Manual; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, (1989) (Maniatis) and by T. J.  
20 Silhavy, M. L. Bannan, and L. W. Enquist, Experiments with Gene Fusions, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1984) and by Ausubel, F. M. et al., Current Protocols in Molecular Biology, pub. by Greene Publishing Assoc. and Wiley-Interscience (1987).

Materials and methods suitable for the maintenance and growth of  
25 bacterial cultures are well known in the art. Techniques suitable for use in the following examples may be found as set out in Manual of Methods for General Bacteriology (Phillipp Gerhardt, R. G. E. Murray, Ralph N. Costilow, Eugene W. Nester, Willis A. Wood, Noel R. Krieg and G. Briggs Phillips, eds), American Society for Microbiology, Washington, DC (1994)) or by Thomas D. Brock in  
30 Biotechnology: A Textbook of Industrial Microbiology, Second Edition, Sinauer Associates, Inc., Sunderland, MA (1989). All reagents, restriction enzymes and materials used for the growth and maintenance of bacterial cells were obtained from Aldrich Chemicals (Milwaukee, WI), DIFCO Laboratories (Detroit, MI), GIBCO/BRL (Gaithersburg, MD), or Sigma Chemical Company (St. Louis, MO)  
35 unless otherwise specified.

Materials and methods suitable for the transformation and growth of plants are well known in the art. Techniques suitable for use in the following examples may be found as set out in Plant Molecular Biology, A Laboratory Manual



(Melody S. Clark, eds., Springer-Verlag, Berlin, Heidelberg, 1997), Methods in Plant Molecular Biology, A Laboratory Course Manual (Pal Maliga, Daniel F. Flessing, Anthony R. Cashmore, Wilhelm Cruissem, Joseph E. Varner, eds., Cold Spring Harbor Laboratory Press, 1995), and Methods in Molecular Biology, Volume 82, Arabidopsis Protocols (Jose M. Martinez-Zapater, Julio Salinas, eds., Humana Press, Totowa, NJ 1998). All reagents, restriction enzymes and materials used for the growth and maintenance of transgenic plants were obtained from Aldrich Chemicals (Milwaukee, WI), DIFCO Laboratories (Detroit, MI), GIBCO/BRL (Gaithersburg, MD), or Sigma Chemical Company (St. Louis, MO) unless otherwise specified.

The meaning of abbreviations is as follows: "h" means hour(s), "min" means minute(s), "sec" means second(s), "d" means day(s), "mL" means milliliters, "L" means liters.

#### EXAMPLE 1

##### Construction of Plasmids Containing Synthetic Genes for Analogs of *Nephila Clavipes* Spidroin 1 for Expression in *Arabidopsis*

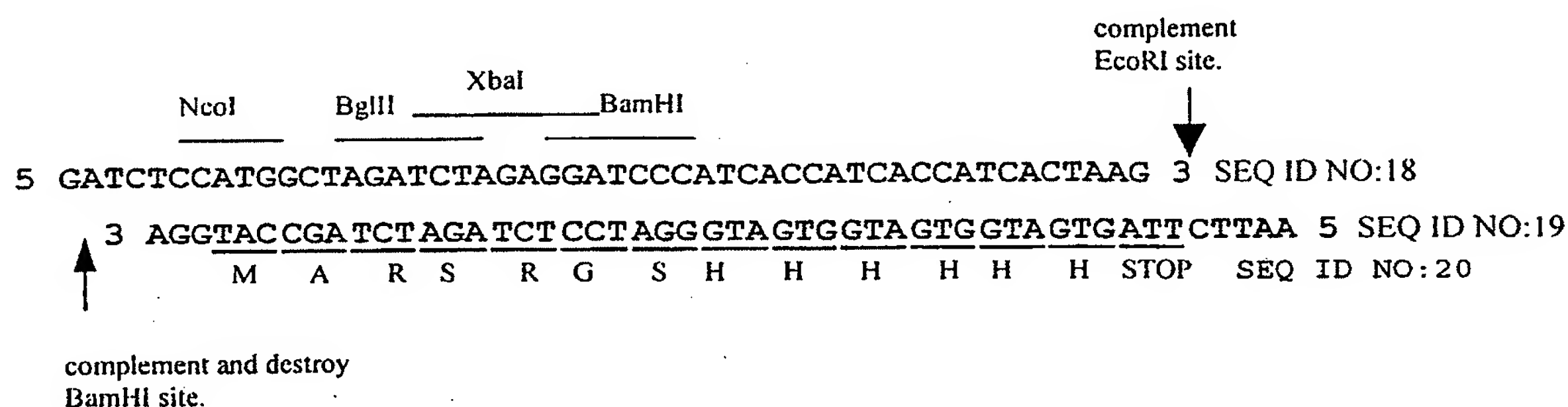
Synthetic genes of 8-mer and 16-mer DP-1B.33 were obtained from the DuPont Company (Wilmington, DE 19898) (WO 9429450). These genes encode for 809 (SEQ ID NO:13) and 1617 (SEQ ID NO:14) amino acid protein sequences, respectively, that represent essential structural element and repetitive pattern in *Nephila clavipes* Spidroin 1. Plasmid pFP717 and pFP723 (fully described in WO 9429450), which carry those synthetic genes, were obtained for these experiments.

To add a start codon at the N-terminus, and a 6-histidine coding sequence followed by a stop codon at C-terminus of the synthetic genes, adapter GYS was made. Oligonucleotide sequences GYS[+] (5' GAT CTC CAT GGC TAG ATC TAG AGG ATC CCA TCA CCA TCA CCA TCA CTA AG 3') (SEQ ID NO:15) and GYS[-] (5' AAT TCT TAG TGA TGG TGA TGG TGA TGG GAT CCT CTA GAT CTA GCC ATG GA 3') (SEQ ID NO:16) were synthesized by standard methods. The oligonucleotides were diluted to 1 µg/µL with TE (10 mM Tris, 1 mM EDTA, pH 8.0) and mixed into a tube in equal volumes. The mixture was boiled for 5 min and then slowly cooled to room temperature. Adapter GYS formed in this process is shown below. The adapter has sticky ends complementary to BamHI and EcoRI digestion sites, respectively, and encodes for a small peptide including a start codon, ARSRGS (SEQ ID NO:17) 6-histidine tag, and a stop codon. It also introduces a few restriction sites such as NcoI, BglI, XbaI, and BamHI. The adapter was cloned into pBluescript-SK(+) (Stratagene, La Jolla, CA) between restriction sites BamHI and EcoRI by T4 ligase (Life



Technologies, Gaithersburg, MD). The resultant plasmid, called pGY001 (Figure 1) was amplified in XL1-Blue *E. coli* cells (Stratagene, La Jolla, CA) and prepared using QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA). The sequence of the adapter was confirmed by standard sequencing.

5



Two µg of Plasmid Pfp717 and Pfp723 were subjected to 37°C restriction digestion of BglII and BamHI for 2 hrs. 8-mer and 16-mer DP-1B.33 genes were separated on a 0.8% agarose gel and purified using QIAquick Gel Extract Kit (Qiagen, Valencia, CA). Two µg of pGY001 was also digested in a 50 µL reaction by the same enzymes. To make dephosphorylated pGY001, 10 µL of dephosphorylation buffer and 2 µL of CIAP (Life Technologies, Gaithersburg, MD) were added to the reaction and filled with water to a final volume of 100 µL. The reaction mixture was placed at 37°C for 30 min and additional 2 µL of CIAP was added for another 30 min incubation. The DNA was cleaned up by using QIAquick PCR Purification Kit (Qiagen, Valencia, CA). 8-mer and 16-mer DP-1B.33 from pFP717 and pFP723 were then cloned into pGY001 between BglII and BamHI sites using T4 ligase, resulting in pGY101 and pGY102, respectively (Figure 2A and 2B). Plasmids (pGY101, pGY102) were amplified in XL1-Blue *E. coli* and purified using QIAprep Spin Miniprep Kit. These two plasmids, contain the coding regions for the 8-mer (in pGY101) and 16-mer (in pGY102) DP-1B.33 with a N-terminal start codon and a C-terminal 6-histidine coding sequence and a subsequent stop codon added. Thus the plasmids contained two complete synthetic genes, DP-1B 8-mer for plants (SEQ ID NO:21) encoding an 818 amino acid residue polypeptide (SEQ ID NO:22) and DP-1B 16-mer for plants (SEQ ID NO:23) encoding a 1626 amino acid residue polypeptide (SEQ ID NO:24). Accuracy of the insertions was confirmed by DNA sequencing.

## EXAMPLE 2

### Construction of Expression Cassettes

To build cassettes with appropriate 5' promoters and 3' terminators (polyadenylation sequences) for constitutive and seed-specific expression of DP-1B genes, plasmids pML63 and pCW109 were provided by DuPont Agricultural Products (Wilmington DE, 19898). pCW109 is fully described in U.S. 5,955,650 and WO 94/11516. Vector pML63 contains the *uidA* gene (which encodes the GUS enzyme) operably linked to the CaMV35S promoter and 3' NOS sequence. pML63 is modified from pMH40 to produce a minimal 3' NOS terminator fragment. pMH 40 is described in WO 98/16650, the disclosure of which is hereby incorporated by reference. Using standard techniques familiar to those skilled in the art, the 770 base pair terminator sequence contained in pMH40 was replaced with a new 3' NOS terminator sequence comprising nucleotides 1277 to 1556 of the sequence published by Depicker et al. (1982, *J. Appl. Genet.* 1:561-574).

As shown in Figure 3A, pML63 includes a GUS expression cassette with a 5' CaMV 35S/Cab22L promoter and a 3' NOS terminator (35S/Cab22L Pro::GUS::NOS Ter). To replace GUS with DP-1B.8P, pML63 and pGY101 were digested by restriction the enzymes NcoI and EcoRI. The DNA fragment containing DP-1B.8P from pGY101 was cloned into pML63 by the method described earlier. The resultant plasmid was named pGY201 and contained an expression cassette of 35S/Cab22L Pro::DP-1B.8P::NOS Ter. The DP-1B.16P was also substituted for GUS in pML63, in which pGY102 was used instead of pGY101. The plasmid containing an expression cassette of 35S/Cab22L Pro::DP-1B.16P::NOS Ter was designated as pGY202. The detailed structures of both pGY201 and pGY202 are shown in Figure 4A and 4B.

Sequence of pCW109 indicates that it contains an empty expression cassette with a 5'  $\beta$ -conglycinin promoter and a 3' Phaseolin terminator (Figure 3B). To insert DP-1B.8P into polylinker region immediately downstream  $\beta$ -conglycinin promoter, pCW109 and pGY101 were digested with restriction enzymes NcoI and KpnI, and then the DNA fragment containing DP-1B.8P from pGY101 was cloned into pCW109 between restriction sites of these two enzymes. The new plasmid was named pGY211 and contained an expression cassette consisting of  $\beta$ -conglycinin Pro::DP-1B.8P::Phaseolin Ter (Figure 5A). To limit the restriction sites available in the polylinker, 1  $\mu$ g of pGY211 was digested in a 30  $\mu$ L reaction mixture with restriction enzymes EcoRI and XhoI at 37°C for 2 hrs. Then, 2  $\mu$ L of 2.5 mM dNTP, 17  $\mu$ L water, and 1  $\mu$ L Klenow fragment were added to the reaction mixture, and incubated for 10 min at room temperature

to make blunt ends. The reaction was cleaned up by using QIAquick PCR Purification Kit. The new plasmid was obtained by self-ligation of one tenth of the reaction. To make more restriction sites available in regions flanking the expression cassette, the HindIII fragment from the plasmid, containing the entire expression cassette, was cloned into the HindIII site of pBluscript SK(+) in a positive orientation. This plasmid was designated pGY213 (Figure 5B) and its orientation was confirmed by restriction digestion patterns.

### EXAMPLE 3

#### Construction Of Binary Vector-Based Plasmids

The binary vector pZBL1 was provided by DuPont Agriculture Products (Wilmington, DE 19898) and is fully described in U.S. 5,968,793 and is available from the American Type Culture Collection (ATCC 209128). The vector includes a kanamycin resistance gene outside the T-DNA region for bacteria selection, and a NPTII gene expression cassette (NOS Pro::NPTII::OCS Ter) inside the T-DNA region, between sequences of the right border (RB) and the left border (LB), for kanamycin resistance selection of plant cells (Figure 6). All plasmids described in this example were generated in XL1-Blue *E. coli* cells except where mentioned.

To construct binary vector-based plasmids for constitutive expression of DP-1B proteins, plasmid pGY201 and pGY202 were digested by restriction enzymes XbaI and SalI. DNA fragments containing the DP-1B.8P and DP-1B.16P expression cassettes were isolated and inserted into the binary vector pZBL1 between restriction sites XbaI and SalI of the polylinker region, upstream of the NPTII expression cassette, respectively. The insertion resulted in plasmids pGY401, harboring an expression cassette 35S/Cab22L Pro::DP-1B.8P::NOS Ter, and pGY402, harboring an expression cassette 35S/Cab22L Pro::DP-1B.16P::NOS Ter. Structures of both plasmids are detailed in Figure 7A and 7B. Their sequences were confirmed by digestion of unique restriction sites.

Plasmid pGY411 was constructed for seed-specific expression of DP-1B.8P protein using a similar approach as described above. The DNA fragment containing DP-1B.8P expression cassette was obtained from pGY213 by digesting with restriction enzyme EcoRI and SalI and inserted into pZBL1 between these two sites. To make a construct for seed-specific expression of DP-1B.16P, pGY412 was constructed by substitution of the DP-1B.16P coding region (a DNA fragment from restriction site KpnI to BglII in pGY102) for the DP-1B.8P coding region (a DNA fragment between the same restriction sites in pGY411). DNAs for both plasmids were amplified in STBII *E. coli* cells to avoid DNA rearrangement, and the constructs were confirmed by digestion of unique restriction sites. As shown in Figure 8A and 8B, pGY411 and pGY412 include

seed-specific expression cassettes consisting of  $\beta$ -conglycinin Pro::DP-1B.8P::Phaseolin Ter and  $\beta$ -conglycinin Pro::DP-1B.16P::Phaseolin Ter, respectively. The plasmids are summarized in Table 1.

#### EXAMPLE 4

##### 5                    Agrobacterium-Mediated *Arabidopsis* Transformation Agrobacterium transformation

To prepare competent agrobacterial cells, a colony of C58C1(pMP90) agrobacterium strain (Koncz et al., *Mol. Gen. Genet.*, (1986) 204 (3), 383-396) were grown in 1 L YEP media, which includes 10 g Bacto peptone, 10 g yeast  
 10        extract, and 5 g NaCl, until an OD<sub>600</sub> of 1.0. The culture was chilled on ice and the cells were collected by centrifugation. The competent cells were resuspended in ice cold 20 mM CaCl<sub>2</sub> solution and stored in -80°C in 0.1 mL aliquots.

A freeze-thaw method was used to introduce pGY401, pGY402, pGY411, and pGY412 into agrobacteria. At first, 1  $\mu$ g plasmid DNA from each of these  
 15        constructs was added to the frozen aliquoted agrobacterial cells. The mixture was thawed at 37°C for 5 min, added to 1 mL YEP medium, and then gently shaken at 28°C for 2 hrs. Cells were collected by centrifugation and grown on a YEP agar plate containing 25 mg/L gentamycin and 50 mg/L kanamycin at 28°C for 2 to 3 days. Agrobacterial transformants were confirmed by minipreparation and  
 20        restriction enzyme digestion of plasmid DNA by routine methods, except that lysozyme (Sigma, St. Louis, MO) was applied to the cell suspension before DNA preparation to enhance cell lysis. An empty binary vector pZBL1 was also introduced into agrobacteria as a control.

##### *Arabidopsis* transformation

25                *Arabidopsis thaliana* was grown to bolting in 3" square pots of Metro Mix soil (Scotts-Sierra, Maryville, OH) at a density of 5 plants per pot, under a controlled temperature of 22°C and an illumination of 16 hrs light/8 hrs dark. Plants were decapitated 4 days before transformation. Agrobacteria carrying pZBL1(control), pGY401, pGY402, pGY411, or pGY412 plasmids were grown in  
 30        LB medium (1% bacto-tryptone, 0.5% bacto-yeast extract, 1% NaCl, pH 7.0) containing 25 mg/L gentamycin and 50 mg/L kanamycin at 28°C, until the culture reached an OD<sub>600</sub> value of 1.2. Cells were collected by centrifugation and resuspended in infiltration medium (1/2 x MS salt, 1 x B5 vitamins, 5% sucrose, 0.5 g/L MES, pH 5.7, 0.044  $\mu$ M benzylaminopurine) to OD<sub>600</sub> of approx. 0.8.

35                A vacuum infiltration method was employed to transfect the *Arabidopsis* plants with the agrobacterium strains which carried the five binary vector-based plasmids described above. Briefly, a 500 mL Magenta Box was filled with infiltration medium suspension of agrobacterium, and covered with a 3" square



pot containing 5 *Arabidopsis* plants in an upside-down position, so that the entire plant was submerged in the suspension. The assembly was placed in an Isotemp Vacuum Oven model 281 (Fisher Scientific, Pittsburgh, PA)) and subjected to infiltration for 5 min under 30 mm Hg vacuum. At least 3 pots of plants were infiltrated by each of the agrobacterium strains. Infected plants were then laid on their sides in a Saran wrap sealed flat and incubated overnight at room temperature. The transfected *Arabidopsis* plants were grown to maturation under normal condition (22°C, 16 hrs light/8 hrs dark). Seeds from the transformed plants are defined as T1 seeds. T1 seeds were collected from plants in each pot, dried for one week, and stored at room temperature.

### EXAMPLE 5

#### Expression of DP-1B Proteins in *Arabidopsis*

##### Selection of *Arabidopsis* transformants

To select transformants, 1,000 T1 seeds were sterilized in 1 mL of 50% Clorox® (Chloral is ~10% bleach) and 0.02% Triton X-100 solution for 7 min, followed by 5 rinses in sterile distilled water. Seeds were resuspended in 2 mL of 0.1% agarose and spread on the top of a 90 x 20 mm plate containing primary selective medium (1xMS salt, 1xB5 vitamins, 1% sucrose, 0.5 mg/mL MES, pH 5.7, 30 µg/mL kanamycin, 100 µg/mL carbenicilin, 10 µg/mL benomyl, and 0.8% phytagar). After cold treatment at 4°C for 3 days, seeds were allowed to germinate for one week at 22°C under continuous illumination. Due to expression of the NTPII gene, all transformant seeds, which usually account for approximately 1% of the seed collection, germinated and grew into green seedlings. However, non-transformant seeds either did not germinate or their seedlings quickly became bleached. Healthy transformant seedlings, defined as T1 plants, were selected and grown on another 90 x 20 mm plate containing secondary selective medium, which had the same components as the primary selective medium except 15% phytagar. Transformants were grown for one week to enhance root development. Finally, the seedlings were transferred to individual 1" square pots of Metro Mix soil and grown to maturation at 22°C and 16 hrs light/8 hrs dark cycle. T2 seeds produced by T1 plants were collected from each individual plant and stored separately.

All the T1 seed collections of pZBL1, pGY401, pGY402, pGY411, and pGY412 were subject to the transformant selection described above. This process resulted in 22 transgenic plants for pZBL1, 44 for pGY401, 69 for pGY402, 21 for pGY411, and 29 for pGY412.

Examination of DP-1B protein expression

T1 transgenic plants carrying the pGY401 and pGY402 constructs were selected and grown in soil until bolting as described above. Half of a healthy leaf (approximately 20 mg of leaf tissues) from each plant was ground with 50  $\mu$ L protein extract buffer (50 mM Tris-HCl, pH 8.0, 12.5 mM  $MgCl_2$ , 0.1 mM EDTA, 2 mM DTT, 5% glycerol) in 1.5 mL ice-cold Eppendorf tubes. The mixtures were centrifuged and the supernatants were collected as leaf protein extracts for examination of constitutively expressed DP-1B protein. Seed protein extracts were prepared from T2 seeds carrying pGY411 and pGY412 constructs, which had been harvested from the selected T1 transgenic plants as described above. 100 to 200 seeds from each transgenic plant were extracted in 400  $\mu$ L of protein extract buffer. Seed protein extracts were used to examine seed-specific expression of DP-1B protein. Total protein concentrations in these extracts were determined by using Bio-Rad Protein Assay Reagent (Bio-Rad, Hercules, CA).

The protein immuno-blot assay described in Current Protocols in Molecular Biology (F. M. Ausubel et al., ed, Wiley Interscience) was employed to determine expression of DP-1B protein. Proteins in leaf protein extract or seed protein extract were separated in a mini-polyacrylamide gel (5% stack gel and 10% separate gel) using a Bio-Rad mini-gel electrophoresis apparatus. Using a Pharmacia-LKB 2117 multiphor II (Amersham Pharmacia Biotech, Piscataway, NJ), proteins in the gel were transferred to a 0.2  $\mu$ M nitrocellulose membrane (Schleicher & Schuell, Keene, NH) for 1 hr at 0.8 mA/cm<sup>2</sup> using a semi-dry transfer method recommended by the manufacturer. One liter of semi-dry western transfer buffer included 2.93 g glycine, 5.81 g Tris, 0.375 g SDS, and 200 mL methanol. The nitrocellulose membrane was blocked with 5% non-fat milk TTBS (0.1% Tween-20, 2.42 g Tris, 29.2 g NaCl, pH 7.5), incubated in the primary antibody-TTBS solution for 3 hrs, and then in TTBS containing anti-rabbit IgG HRP-conjugate (Promega, Madison, WI) for 1 hr. Protein-antibody interaction on the membrane was detected by a chemiluminescent substrate solution, which consisted of 100 mM Tris-HCl buffer (pH 8.5) containing 0.2 mM P-coumaric acid, 2.5 mM 3-aminophthalhydrazide and 0.01% H<sub>2</sub>O<sub>2</sub>. The results were visualized by exposure to X-ray film.

To examine expression of DP-1B proteins, 10  $\mu$ L leaf protein extracts from pGY401 and pGY402 transgenic *Arabidopsis* and 10  $\mu$ L seed protein extracts from pGY411 and pGY412 transgenic *Arabidopsis* were subjected to protein immuno-blot assay. Ten  $\mu$ L leaf and seed protein extracts from pZBL1 transgenic *Arabidopsis* were also used as controls. The primary antibody, DP-1B Abs, was obtained from DuPont, the preparation of which is fully described in

WO 9429450. These antibodies recognize the highly conserved sequence CGAGQGGYGGLGSGGAGR (SEQ ID NO:25) in the DP-1B molecule, and were used in a 1:1,000 dilution. Figure 9A illustrates the results from the protein immuno-blot assay, indicating that the 64 kD DP-1B.8P and 127 kD DP-1B.16P proteins were produced and accumulated in leaf tissues of pGY401 and pGY402 transgenic *Arabidopsis*, and that the both proteins were also produced and accumulated in seeds of pGY411 and pGY412 transgenic *Arabidopsis*, respectively. A higher ratio of smaller fragment of DP-1B.16P proteins accumulated in leaves of pGY402 plants and seeds of some pGY412 plants indicating that production of DP-1B protein in *Arabidopsis* prefers the 8-mer to the 16-mer. Using this assay, 163 transgenic *Arabidopsis* with kanamycin-resistance phenotype (44 for pGY401, 69 for pGY402, 21 for pGY411, and 29 for pGY412) were examined for DP-1B expression. Only 25 pGY401 plants (57%), 4 pGY402 plants (6%), 4 pGY411 plants (19%), and 7 pGY412 plants (24%) produced and accumulated DP-1B protein products with expected molecular masses.

**TABLE 1**  
A Summary for Plasmid Constructs

Construct	Recipient	Donator	Insertion	Usage
pGY001	pBS-SK(+)	-	Adapter GYS	Adapter
pGY101	pGY001	pFP717	8xDP-1B.33	DP-1B.8P
pGY102	pGY001	pFP723	16xDP-1B.33	DP-1B.16P
pGY201	pML63	pGY101	DP-1B.8P	35S/Cab22L Pro:: DP-1B.8P::NOS Ter
pGY201	pML63	pGY102	DP-1B.16P	35S/Cab22L Pro:: DP-1B.16P::NOS Ter
pGY211	pCW109	pGY101	DP-1B.8P	Beta-conglycinin Pro:: DP-1B.8P::Phaseolin Ter
pGY213	pBS-SK(+)	pGY211	DP-1B.8P	Beta-conglycinin Pro:: DP-1B.8P::Phaseoline Ter
pGY401	pZBL1	pGY201	35S Pro::DP-1B.8P:: NOS Ter	Constitutive expression of DP-1B.8P in Arabidopsis
pGY402	pZBL1	pGY202	35S Pro::DP-1B.16P:: NOS Ter	Constitutive expression of DP-1B.16P in Arabidopsis
pGY411	pZBL1	pGY213	Cong Pro::DP-1B.8P:: Pha Ter	Seed-specific expression of DP-1B.8P in Arabidopsis
pGY412	pGY411	pGY102	Cong Pro::DP-1B.16P:: Pha Ter	Seed-specific expression of DP-1B.8P in Arabidopsis

TABLE I  
A Summary for Plasmid Constructs

pLS3	pZBL102	pGY213	Cong Pro::DP-1B.8P:: Pha Ter	Expression of DP-1B.8P in Soy somatic embryos
pGY220	pGY213	pGY412	Cong Pro::DP-1B.16P:: Pha Ter	Beta-conglycinin Pro:: DP-1B.16P::Phaseolin Ter
pLS4	pZBL102	pGY220	Cong Pro::DP-1B.16P:: Pha Ter	Expression of DP-1B.16P in Soy somatic embryos

The remaining transgenic *Arabidopsis*, which had been selected by their antibiotics-resistance phenotypes, belonged to the following three categories:

(1) Plants showed no visible accumulation of DP-1B protein in the assay;

5 (2) Plants expressed DP-1B proteins but were sterile or died before maturation;

(3) Plants accumulated DP-1B protein with wrong molecular mass or/and multiple dominant products. The fact that few transgenic plants successfully produced DP-1B proteins reflects the difficulty in getting expression of SLP's in plants, possibly due to high repetitive and high glycine/alanine enriched nature of spider

10 silk.

Anti-His (C-term)-HRP (Invitrogen, Carlsbad, CA) was also used as a primary antibody in the protein immuno-blot assay. Because 6 x histidine tag was built into C-terminus of DP-1B protein in all constructs, the anti-His tag conjugate enabled us to determine the quality and estimate the yield of DP-1B proteins

15 conveniently. When using this antibody for immuno-blot, the secondary antibody was not necessary and protein-antibody interaction could be detected directly by chemiluminescent reagents.

To determine the quality of DP-1B proteins produced in transgenic *Arabidopsis*, leaf or seed protein extracts from those 40 plants, which

20 demonstrated expected expression of DP-1B proteins, were subjected to immuno-blot assays. Anti-His (C-term)-HRP was used in a 1:4,000 dilution as the primary antibody. Figure 9B illustrates the results from this assay. The results indicated that expressed DP-1B proteins in those plants had not only the correct molecular masse but also the complete C-termini, since their C-terminal His-tags were

25 recognized by anti-His (C-term)-HRP. Shorter fragment ladders of DP-1B.16P protein, which were detected by DP-1B Abs in some of protein extracts such as 402(92), 402(94), and 412(41) of Figure 9A, were not recognized by the His-tag Ab, suggesting that some premature terminations might have occurred during the translation of DP-1B.16P. When interacting with seed proteins, anti-His

30 (C-term)-HRP also recognized a few smaller protein molecules, as shown in the



right panel of Figure 9B. Since these proteins could also be distinguished from the control, it is assumed that they were seed proteins rather than products of transgenes.

In a similar immuno-blot assay using Anti-His (C-term)-HRP, a 14 kD recombinant protein with 6xHis tag at the C-terminus, which was produced in *E. coli* and purified through affinity columns, was used as a standard protein. By comparing signals of the standard protein and protein extracts from the transgenic plants, yields of DP-1B protein in most of those 40 plants were estimated. Yields of DP-1B.8P protein in leaves of pGY401 transgenic plants were between 0.01% and 1.65% of total soluble leaf protein (approximately between 0.002% and 0.33% of dry weight), which represented an average yield of 0.34% of total soluble leaf protein (approximately 0.07% of dry weight). Yields of DP-1B.16P protein in leaves of pGY402 transgenic plants were between 0.01% and 0.06% of total soluble leaf protein (approximately between 0.002% and 0.01% of dry weight), which represented an average yield of 0.03% of total soluble leaf protein (approximately 0.006% of dry weight). Yields of DP-1B.8P protein in seeds of pGY411 transgenic plants were between 1% and 1.4% of total soluble seed protein (approximately between 0.2% and 0.28% of dry weight), which represented an average yield of 1.2% of total soluble seed protein (approximately 0.24% of dry weight). Yields of DP-1B.16P protein in seeds of pGY412 transgenic plants were between 0.5% and 1% of total soluble seed protein (approximately between 0.1% and 0.2% of dry weight), which represented an average yield of 0.78% of total soluble seed protein (approximately 0.16% of dry weight). A summary of the expression results is shown in Table 2.

**TABLE 2**  
DP-1B Yields in Transgenic *Arabidopsis* Plants

Transgene	Product	Examined Tissue	Yield Range (%)		Average Yield (%)	
			of total soluble protein	of dry weight	of total soluble protein	of dry weight
pGY401	DP-1B.8P	Leaves	0.01-1.65	0.002-0.33	0.34	0.07
pGY402	DP-1B.16P	Leaves	0.01-0.06	0.002-0.01	0.03	0.006
pGY411	DP-1B.8P	Seeds	1-1.4	0.2-0.28	1.2	0.24
pGY412	DP-1B.16P	Seeds	0.5-1	0.1-0.2	0.78	0.16

After an extended screening of pGY401 transgenic *Arabidopsis*, one plant was identified which accumulated 65 kD DP-1B.8P protein up to 9.2% of total soluble leaf protein (approximately 1.8% of dry weight), not shown in Table 2. These results suggested that, in general, seed-specific expressions (pGY411 and

pGY412) led to higher levels of both DP-1B.8P and DP-1B.16P proteins in seeds than constitutive expression (pGY401 and pGY402) in leaves.

#### Confirmation of T-DNA insertion into *Arabidopsis* genomes

During *Arabidopsis* transformation, the entire T-DNA sequence, which  
5 included NPTII expression cassette and DP-1B.8P or DP-1B.16P expression  
cassette was inserted into the plant genome. To further relate the expression of  
DP-1B proteins in those 40 transgenic *Arabidopsis* to the transgenes, polymerase  
chain reaction (PCR) was employed to detect a DNA fragment within the T-DNA  
region from genomic DNA of those plants. For this purpose, 2 leaves  
10 (approximately 100 mg) were collected from each transgenic *Arabidopsis*. DNA  
was then isolated using DNeasy Plant Mini Kit, following a protocol provided by  
kit manufacturer (Qiagen, Valencia, CA), and 50  $\mu$ L of a DNA solution was  
obtained. The DNA concentration and purity of each preparation was estimated  
by measuring OD<sub>260</sub> and OD<sub>280</sub> values in a Beckman DU640 Spectrophotometer  
15 (Beckman Instruments, Fullerton, CA). Since direct amplification of DP-1B  
coding regions was difficult due to its highly repetitive nature, primer NPTII-F2  
(5' GCT,CGA,CGT,TGT,CAC,TGA,AG 3') (SEQ ID NO:26) and NPTII-R2  
(5' TCG,TCC,AGA,TCA,TCC,TGA,TC 3')(SEQ ID NO:27) were synthesized by  
standard means and used to amplify a 240 bp segment of the NPTII gene. One  
20 25  $\mu$ L PCR reaction included 1  $\mu$ L DNA, 2.5  $\mu$ L 10xPCR reaction buffer (Life  
Technologies, Gaithersburg, MD), 0.25 mM each of dNTP, 2 mM MgCl<sub>2</sub>,  
10 pmole primer for NPTII-F2, 10 pmole primer for NPTII-R2, and 1.25 units of  
Taq DNA polymerase (Life Technologies, Gaithersburg, MD). Reactions were  
conducted on a GeneAmp PCR System 960 (Perkin-Elmer, Norwalk, CT) for  
25 35 cycles of 45 sec at 94°C, 45 sec at 58°C, and 45 sec at 72°C, and then  
separated on an electrophoretic agarose gel containing ethidium bromide. Results  
were visualized under UV light. Analysis of the gel indicated that the T-DNAs  
had been integrated into genomic DNAs of all 40 transgenic *Arabidopsis* as  
expected. The results are shown in Figure 9C. Because the DNA sample for the  
30 control was prepared from a pZBL1 transgene plant, which carries NPTII gene but  
not DP-1B gene, a 240 bp NPTII fragment was amplified from it by PCR.  
Therefore, the DNA sample from wild type (WT) *Arabidopsis* was used in this  
assay as a negative control.

#### Demonstration of transgene heritability

35 To test transgene heritability, two transgenic *Arabidopsis* plants were  
chosen containing each of pGY401, pGY402, pGY411, and pGY412 constructs.  
T2 seeds were cold-treated for 3 days and then germinated on primary selective  
medium for 10 days. Thirty healthy kanamycin resistance T2 seedlings, which

were expected to contain the transgene, were transferred and grown in Metro Mix soil under the conditions described above. Protein extracts were prepared from leaves of bolting plants of pGY401 and pGY402 and seeds of mature plants from the pGY411 and pGY412 transformants. An immuno-blot assay, using a polyclonal antibody against the highly conserved peptide sequence of DP-1B protein (DP-1B Abs), demonstrated that DP-1B.8P and DP-1B.16P proteins were produced and accumulated in T2 progenies of the transgenic plants in a tissue-specific manner (Figure 10A). Smaller peptide fragments of DP-1B.16P protein also accumulated in T2 plants of 402(92), 402(94), and 412(41) in similar patterns as seen in their T1 parents.

DNA was also isolated from leaves of these T2 progenies. PCR amplification of 240 bp NPTII fragment was carried out for each DNA sample, following the protocol described above. DNA samples from wild-type (WT) *Arabidopsis* was used as a negative control since the control DNA of pZBL1 transgenic plant contained the NPTII sequence. PCR reactions were then subjected to electrophoresis on an agarose gel containing ethidium bromide. The gels were visualized under UV light (Figure 10B), and indicated that the genomes of all these T2 progenies still carried the transgenes.

Along with examining transgenes expression, the germination and development of these T2 plants were also analyzed. A comparison of the T2 plants with the control plants (pZBL1) during their growth showed no phenotypic abnormality among T2 plants in spite of expression of transgenes.

In conclusion, these results demonstrated that the DP-1B gene, which was introduced into the *Arabidopsis* genome using constructs pGY401, pGY402, pGY411, and pGY412, were heritable and stable through sexual reproduction.

#### EXAMPLE 6

##### Construction of Plasmids Containing Synthetic Genes for Analogs of *Nephila Clavipes* Spidroin 1 for Expression in Soy Somatic Embryos

Plasmid pZBL102 was provided by DuPont Agricultural Products (Wilmington, DE 19898). This plasmid was used to make constructs for DP-1B protein expression in soy somatic embryos. This pSP72 (Promega, Madison, WI)-based plasmid contains an hygromycin B phosphotransferase (HPT) gene directed by T7 promoter (T7 Pro::HPT::T7 Ter) for hygromycin B resistance in bacterium and an expression cassette of 35S Pro::HPT::NOS Ter for hygromycin B resistance in plant cells, as shown in Figure 11A. Because of the highly repetitive nature of the DP-1B coding sequences, all plasmids in this example were generated in STBII *E. coli* cells.



To make a construct for expression of DP-1B.8P protein in soy somatic embryos, plasmid pZBL102 was digested with NotI and Sall. The linearized vector was separated from a short NotI/Sall DNA fragment on an agarose gel and purified using QIAquick Gel Extract Kit. Using the same method, plasmid pGY213 was also digested by NotI and Sall and a 4357 base pair DNA fragment containing a seed-specific expression cassette consisting of  $\beta$ -conglycinin Pro::DP-1B.8P::Phaseolin Pro was isolated. This DNA fragment was ligated with the linearized pZBL102 between the NotI and Sall sites in an orientation which was the same as that for the 35S Pro::HPT::NOS Ter expression cassette. The new construct was designated pLS3. Its structure is shown in Figure 12A.

Construction of a plasmid for expression of DP-1B.16P protein in soy somatic embryos required a modified plasmid pGY412. For this purpose, the DNA fragment between KpnI (1282) and EcoRI (1330) sites of pGY412 was replaced by a short sequence that only included a SmaI site. This modified pGY412 was then digested with Sall and NcoI, and a DNA fragment containing the DP-1B.16P coding region and the Phaseolin terminator sequence was isolated and ligated into pGY213 between Sall and NcoI sites. This fragment was thus substituted for the DP-1B.8P coding region and resulted in plasmid pGY220. Figure 11B shows structure of plasmid pGY220, which contains a seed-specific expression cassette consisting of  $\beta$ -conglycinin Pro::DP-1B.16P::Phaseolin Ter.

In a similar manner plasmid pGY220 was digested with NotI and Sall. A 6774 base pair DNA fragment containing a seed-specific expression cassette consisting of  $\beta$ -conglycinin Pro::DP-1B.16P::Phaseolin Ter was isolated and ligated with the linearized pZBL102 between the NotI and Sall sites. The new plasmid, pLS4, was almost identical to pLS3, except it contained the DP-1B.16P coding region instead of the DP-1B.8P region. Its structure is shown in Figure 12B.

#### EXAMPLE 7

##### Transformation and Expression of DP-1B Gene in Soy Somatic Embryos

##### Somatic Embryonic Cell Transformation by Particle-Gun Bombardment

Plasmids pLS3 and pLS4 were used in soy somatic embryonic cell transformation in order to express the 8-mer and 16-mer DP-1B protein, respectively. Prior to transformation, both plasmids were amplified and purified from STBII *E. coli* cells on a large scale. STBII cells carrying with pLS3 or pLS4 were grown in 500 mL of LB-hygromycin broth (10 g/L Bacto tryptone, 5 g/L yeast extract, 5 g/L NaCl, 150 mg/L hygromycin B), at 37°C overnight, and collected by centrifugation. The cells were then resuspended in 6 mL of solution I (25 mM Tris pH 7.5, 10 mM EDTA, 15% sucrose, 2 mg/mL lysozyme), lysed by



adding 12 mL of solution II (0.2 M NaOH, 1% SDS), and then neutralized by adding 7.5 mL of 3 M NaAc, pH 4.6. Supernatant of the lysate was collected by centrifugation, and subjected to 50 µg RNase A treatment at 37°C for 30 min, phenol/chloroform extraction, and ethanol precipitation. The DNA pellet was resuspended in 1 mL H<sub>2</sub>O and precipitated again by mixing with 1 mL 1.6 M NaCl and 2 mL 13% PEG-800. Pure DNA was washed with 70% ethanol and resuspended in H<sub>2</sub>O with a final concentration of 1 µg/µL.

Two week-old suspension cultures of soy somatic embryonic cells Asgro 2872/821 were transformed with plasmid pLS3 and pLS4 using particle gun bombardment (U.S. 5,955,650). The bombardment was carried out in a DuPont Biolistic PDS1000/HE instrument (helium retrofit) at 1100 psi membrane rupture pressure and 27-28 in. Hg chamber vacuum. Ten plates of cells were transformed for each construct, by double bombardments. Following bombardment, cells were incubated for 11 days in SB172 (4.6 g/L Duchefa MS salt, 1 mL/L 1,000x B5 vitamins, 10 mg/L 2,4-D, 60 g/L sucrose, 667 mg/L asparagine, pH 5.7), and transformant clones were selected over the next 2 months in SB172 containing 50 mg/L hygromycin B. Sixty pLS3 and thirty pLS4 transformant clones were chosen for further maturation of embryonic tissue by sequentially culturing them following a three-step schedule: (1) 1 week on SB166 (34.6 g/L Gibco/BRL MS salts, 1 mL/L 1,000x B5 vitamins, 60 g/L maltose, 750 mg/L MgCl<sub>2</sub> hexahydrate, 5 g/L activated charcoal, 2 g/L gelrite, pH 5.7); (2) 3 weeks on SB103 (as same as SB166 but without activated charcoal); (3) 2 weeks on SB148 (as same as SB103 except that 7 g/L agarose were substituted for 2 g/L gelrite). During the course of experiment, tissue cultures in both liquid and solid media were maintained under a controlled condition of 26°C, 16:8 hr day/night photoperiod, and light intensity of 30-35 µE/m<sup>2</sup>s.

#### Examination of DP-1B Protein Expression in Soy Somatic Embryos

Mature soy somatic embryo clumps were transformed with pLS3 and pLS4. Each clump represented an independent transformation event and displayed a hygromycin B resistance phenotype. Because it is believed that entire bombarded plasmid will integrate into chromosomes of embryonic cells in most transformation events, the seed-specific DP-1B expression cassettes of pLS3 and pLS4 should be present in those chromosomes and therefore express DP-1B protein.

To examine DP-1B protein expression in the transgenic soy somatic embryos, the protein extracts were prepared from approximately 200 mg of the pLS3 and pLS4 transgenic embryonic tissues by grinding in 200 µL protein extract buffer in a biopulverizer (FastPrep FP120, BIO101, Vista, CA).

Supernatants were collected by centrifugation and protein concentrations were determined by using Bio-Rad Protein Assay Reagent. Wild-type soy embryonic tissue was employed as a control for the experiment. These protein extracts were used in protein immuno-blot assay to determine qualities and quantities of DP-1B protein expression in the transgenic soy embryonic tissues, following a method described in the *Arabidopsis* transformation section.

For the immuno-blot assay, the soluble proteins from 10  $\mu$ L of embryonic protein extract were separated by SDS-PAGE, transferred to nitrocellulose membrane, and then detected using DP-1B Abs. Because of cross-reactions between the antibodies and the embryonic proteins many non-DP-1B proteins were detected by the antibodies from protein extracts of the transformants and control. However, the results still clearly indicated that the 65 kD DP-1B.8P protein had accumulated to significant levels in seven pLS3 embryonic transformants. No detectable 127 kD DP-1B.16P protein had accumulated in any of 30 pLS4 transformants. Additionally, a few of DP-1B.8P transgenic soy somatic embryos also accumulated smaller proteins which were recognized by the DP-1B Abs, suggesting possible DNA recombination or other molecular modifications during transgene expression. Expression levels of DP-1B.8P in those seven pLS3 embryonic transformants were estimated by an immuno-blot assay, probing with anti-His (C-term)-HRP Ab, as described previously. The results are summarized in Table 3.

**TABLE 3**  
DP-1B Yields in Transgenic Soy Embryos

Transgene	Product	Examined Tissue	Yield Range (%)		Average Yield (%)	
			of total soluble protein	of dry weight	of total soluble protein	of dry weight
pLS3	DP-1B.8P	Embryos	0.54-1.64	0.22-0.66	1.0	0.4
pLS4	DP-1B.16P	Embryos	None	None	None	None

As shown in Table 3, the expression levels of DP-1B.8P ranged from 0.54% to 1.64% of total soluble soy embryonic proteins (approximately from 0.22% to 0.66% of dry weight), with an average yield of 1.0% of total soluble soy embryonic proteins (approximately 0.4% of dry weight). (Author's note: assume that 40% of dry weight is protein and all proteins are soluble in embryonic tissue.)

To overcome the antibody-native protein cross-reactions, the protein extracts of the transgenic and wild-type (control) soy somatic embryonic tissues were partially purified using a Ni-NTA Spin Kit (Qiagen, Valencia, CA), prior to immuno-blot assay. Briefly, the protein extract made from 200 mg embryonic

tissue was diluted by adding 400  $\mu$ L lysis buffer and then loaded onto a pre-equilibrated Ni-NTA spin column. DP-1B protein in the extract was bound to the column by a 2 min centrifugation at 700 x g, washed twice with 600  $\mu$ L wash buffer, and finally eluted with 200  $\mu$ L elution buffer. Twenty  $\mu$ L of the partially purified protein extract was run on a SDS-PAGE and examined by immuno-blot assay. The assay probed with DP-1B Abs confirmed accumulation of 65 kD DP-1B.8P protein in those 7 selected pLS3 transformants of soy somatic embryos. It also confirmed that no 127 kD DP-1B.16P protein had accumulated to a detectable level in the pLS4 transgenic embryos. The results are shown in Figure 13A. The immuno-blot assay probed with Anti-His(C-term)-HRP further demonstrated that the all of the accumulated DP-1B.8P consisted of full length molecules since their N-terminal 6xHis-tags were recognized (Figure 13B). Additionally the anti-His (C-term)-HRP also recognized a few smaller protein molecules in the embryo protein extracts, which is shown in the right panel of Figure 13B. Since these proteins were also detected from the protein extract of wild-type embryo, it is concluded that they must be native embryo proteins rather than the products of the transgenes.

#### Confirmation of Transgene Insertion into Genomes of Soy Somatic Embryos

It was expected that most of the soy somatic embryonic colonies surviving hygromycin B selection were transgenic embryos, though many of them did not accumulate DP-1B protein. To further demonstrate that DP-1B.8P and DP-1B.16P transgenes did integrate into chromosome of the embryos, DNA samples were prepared from those embryonic tissues and a control wild-type embryo, using DNeasy Plant Mini Kit (Qiagen, Valencia, CA). Preparations used 100 mg embryonic tissue in 100  $\mu$ L DNA solution by following manufacturer's instruction. DNA concentration and purity of each preparation were estimated by measuring OD<sub>260</sub> and OD<sub>280</sub> values in a Beckman DU640 Spectrophotometer. The DNA samples were subjected to PCR reactions, as described earlier. Primer 5' conglycinin-F (5' CCC,GTC,AAA,CTG, CAT,GCC,AC 3') (SEQ ID NO:28) and primer 5' conglycinin-R (5' TAG,CCA,TGG,TTA,GTA, TAT,CTT 3') (SEQ ID NO:29) were used to amplify a 160 bp fragment of the  $\beta$ -conglycinin promoter. The reactions were separated on an agarose gel containing ethidium bromide, and results were visualized under UV light. Results are shown in Figure 13C. Figure 13C indicates the expected DNA products and confirmed the integration of DP-1B transgenes.

## EXAMPLE 8

### PURIFICATION OF DP-1B PROTEIN FROM *Arabidopsis*

#### Homozygous plant selection and large-scale growth

To obtain large amount of start material, homozygous transgenic plant was selected for direct soil growth. T1 seeds are define as seeds collected from transformed flowers. T1 plant is the plant germinated from T1 seed. T2 seeds are collected from T1 plant. When T2 seeds are germinated, the resulting plants are called T2 plants. At first, T2 seeds were collected from the pGY401 transgenic *Arabidopsis* expressing DP-1B.8P protein in leaf tissue up to 9.2% of total soluble protein, as described in Example 5. Since *Arabidopsis*' self-fertilization nature, heterozygous and homozygous progenies respectively represent 50% and 25% of population among the T2 seed collection. These T2 seeds were germinated as T2 plants on the primary selective medium and twelve of them were grown in Metro Mix soil until maturation in a method described earlier. T3 seeds were harvested from each of twelve plants and germinated on the primary selective media separately. Only homozygous T3 seeds could germinate as T3 plants on the selective medium without showing segregation. Therefore, T4 seeds were collected from those homozygous T3 plants for future use.

For larger scale growth, the T4 homozygous seeds prepared above were germinated and grown on top of Metro Mix soil in 20 x 10 inch flats, in a density of approximately 1,000 seeds per flat. To ensure larger rosettes, plants were grown in a 22°C temperature-controlling green house with less than 10 hours natural lighting. The plants were harvested before bolting, treated with liquid nitrogen, and stored in -80°C. DP-1B.8P transgene insertion and protein synthesis in the transgenic plants were confirmed by immunoblot and PCR assays, respectively, as described earlier.

#### Purification of DP-1B.8P protein

A DP-1B protein purification protocol was developed. It utilizes SLP's special precipitation properties to separate DP-1B protein from plant native proteins, as described below:

- (1) Plant rosettes were homogenized in 5 x volume of ice-cold protein extract buffer (50 mM Tris.HCl pH 8.0, 12.5 mM MgCl<sub>2</sub>, 0.1 mM EDTA, 2 mM DTT, 5% glycerol) using a kitchen blender.

Homogenate was filtrated through 6-layers of cheesecloth and then centrifuged at 10,000 x g for 10 min at 4°C. Supernatant was kept as protein extract.

- (2) The concentrated HCl was slowly added into the stirred protein extract until pH 4.7. The extract was kept in 4°C for 30 min and then



centrifuged at 10,000 x g at 4°C for 30 min to remove protein precipitation. The pH value of the supernatant was adjusted back to 8.0 by slowly adding 10 N NaOH. The resulting solution was saved as pH 4.7 supernatant.

- 5 (3) The pH 4.7 supernatant was subjected to heat treatment in a 60°C waterbath for 60 min and then centrifuged at 10,000 x g at 4°C for 30 min to remove protein precipitation. The supernatant was filtered through one layer of 20 µm nylon mesh and saved. The supernatant were named as "60°C Supernatant".
- 10 (4) (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> was slowly added and dissolved into the stirred 60°C Supernatant in an ice-water bath up to 40% saturation. The solution was kept at 4°C overnight and then centrifuged at 10,000 x g at 4°C for 30 min. The supernatant was named and saved as "(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> Supernatant". Protein precipitation was resuspended and dialyzed
- 15 with protein extract buffer, resulted in a DP-1B.8P protein solution in the one fifteenth of original volume.

To examine total protein profiles during the course of purification, protein samples from each step were subjected to SDS-PAGE, which included 20 µL protein extract (Figure 14A, lane 1), 20 µL pH 4.7 supernatant (Figure 14A, lane 2), 20 µL 60°C supernatant (Figure 14A, lane 3), 10 µL (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> precipitation resuspension (Figure 14A, lane 4), and 20 µL (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> supernatant. The gel was stained with coomassie blue staining solution (0.25% coomassie blue R-250, 20% methanol) overnight and then destained in a solution containing 7% acetic acid and 5% methanol (Figure 14A). Due to its unique

20 amino acid composition, DP-1B protein could not be visualized with coomassie blue staining or other conventional staining methods. But Figure 14A does show that each step in the protocol removes a significant amount of plant native proteins from the extract. In (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> precipitation fraction (Figure 14A, lane 4), more than 95% of plant native proteins has been cleaned out.

30 To monitor DP-1B protein purification, an identical SDS-PAGE was carried out. The gel was transferred to a nitrocellulose membrane and subjected to immunoblot assay in a method described earlier. The DP-1B antibody was used as the primary antibody and the anti-rabbit IgG HRP as the secondary antibody. Result in Figure 14B shows that the 64 kD DP-1B.8P protein was present in all

35 examined fractions, except (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> supernatant, during the course of purification. It is extremely enriched in the resuspension of 40% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> protein precipitation (Figure 14B, lane 4). We have also examined pH 6.7 and 60°C protein precipitation fractions, and no DP-1B.8P protein was detected (data

not shown). Thus, DP-1B protein is concentrated into  $(\text{NH}_4)_2\text{SO}_4$  precipitation fraction.

In conclusion, we have developed a simple DP-1B purification protocol that removes more than 95% of plant native proteins while concentrates DP-1B protein. Due to a 6 x histidine tag is attached with C-terminus of DP-1B protein, Ni-column chromatography will possibly further purify the protein to higher purity

CLAIMS

What is claimed is:

1. A method for the production of silk-like proteins in a green plant comprising:

- 5 a) providing a green plant containing a SLP expression cassette having the following structure:

P-SLP-T

wherein:

10 P is a promoter suitable for driving the expression of a silk-like protein gene;

SLP is a transgene encoding a mature silk-like protein; and

T is a 5' terminator;

wherein each of P, SLP and T are operably linked such that expression of the cassette results in translation of the silk-like protein;

- 15 b) growing said green plant under conditions whereby said transgene is expressed and the silk-like protein is produced; and  
c) optionally recovering said silk-like protein.

2. A method according to Claim 1 wherein the promoter is selected from the group consisting of plant constitute and plant tissue specific promoters.

20 3. A method according to Claim 2 wherein the constitutive promoter is selected from the group consisting of CaMV 35S promoter, the nopaline synthase promoter, the octopine synthase promoter, the ribulose-1,5-bisphosphate carboxylase promoter, Adh1-based pEmu, Act1, SAM synthase promoter, and Ubi promoters and the promoter of the chlorophyll a/b binding protein.

25 4. A method according to Claim 2 wherein the tissue specific promoters are those isolated from genes encoding the proteins selected from the group consisting of napin, cruciferin, beta-conglycinin, phaseolin, zein, oleosin, acyl carrier protein, stearyl-ACP desaturase, fatty acid desaturases, glycinin, Bce4, vicilin, and patatin.

30 5. A method according to Claim 1 wherein said transgene expresses a silk-like protein derived from silks produced by *Bombyx mori* or *Nephila clavipes*.

6. A method according to Claim 1 wherein the silk-like protein has the general formula:

$$[(A)_n - (E)_q - (S)_q - (X)_p - (E)_q - (S)_q]_i$$

35 wherein:

A or E are different non-crystalline soft segment of about 10 to 25 amino acids having at least 55% Gly;

S is a semi-crystalline segment of about 6 to 12 amino acids having at

least 33% Ala, and 50% Gly;

X is a crystalline hard segment of about 6-12 amino acids having at least 33% Ala, and 50% Gly; and

wherein,

- 5           n=2, 4, 8, 16, 32, 64, or 128;  
             q=0, 1, 2, 4, 8, 16, 32, 64, or 128;  
             p=2, 4, 8, 16, 32, 64, or 128;  
             i=1-128; and

where  $p \geq n$  or  $q$ .

- 10           7. The silk-like protein of Claim 6 having the formula selected from the group consisting of:  $[(A)_4-(X)_8]_8$ ,  $[(A)_4-(X)_8-(S)]_8$ ,  $[(A)_4-(X)_8-(E)]_8$ ,  $[(A)_8-(X)_8]_8$ ,  $[(A)_4-(S)-(X)_8]_8$ ,  $[(A)_4-(S)_2-(X)_8]_8$ ,  $[(A)_4-(E)-(X)_8-(E)]_8$ ,  $[(A)_4-(E)-(X)_8]_8$ ,  $[(A)_4-(S)-(X)_8-(E)]_8$ , and  $[(A)_4-(S)_2-(X)_8-(E)]_8$ .

8. The silk-like protein of Claim 6 wherein:

- 15           A= SGGAGGAGG;  
             E=GPGQQGPGGY;  
             S=GAGAGY; and  
             X=SGAGAG.

- 20           9. A full length silk-like protein of Claim 6 wherein the protein is a spider silk variant having the general formula:

$[ACGQGGYGGLGXQGAGRGLGGQGAGAnGG]_z$

wherein X=S, G or N;  $n=0-7$  and  $z=1-75$ , and wherein the value of  $z$  determines the number of repeats in the variant protein and wherein the formula encompasses variations selected from the group consisting of:

- 25           (a) when  $n=0$  the sequence encompassing AGRGGLGGQGAGAnGG is deleted;  
             (b) deletions other than the poly-alanine sequence, limited by the value of  $n$  will encompass integral multiples of three consecutive residues;  
             (c) the deletion of GYG in any repeat is accompanied by deletion of GRG in the same repeat; and  
             (d) where a first repeat where  $n=0$  is deleted, the first repeat is preceded by a second repeat where  $n=6$ ; and

- 30           wherein the full-length protein is encoded by a gene or genes and wherein said gene or genes are not endogenous to the *Nephila clavipes* genome.

10. A method according to Claim 1 wherein the silk-like protein is expressed at levels of about 0.1% to about 9.2%

11. A method according to Claim 1 wherein the silk-like protein is



expressed in leaf and seed tissue.

12. A method according to Claim 1 wherein the green plant is a monocot.

13. A method according to Claim 12 wherein the green plant is selected from the group consisting of corn, wheat, barley, oats, sorghum, rice, rye, grasses and banna.

14. A method according to Claim 1 wherein the green plant is a dicot.

15. A method according to Claim 12 wherein the green plant is selected from the group consisting of soybean, rapeseed, sunflower, cotton, tobacco, alfalfa, *Arabidopsis*, sugar beet, sugar cane, canola, millet, beans, peas, flax, and forage grasses.

16. A green plant expressing a silk-like protein having the general formula:

$$[(A)_n - (E)_q - (S)_q - (X)_p - (E)_q - (S)_q]_i$$

Wherein:

A or E are different non-crystalline soft segment of about 10 to 25 amino acids having at least 55% Gly;

S is a semi-crystalline segment of about 6 to 12 amino acids having at least 33% Ala, and 50% Gly;

X is a crystalline hard segment of about 6-12 amino acids having at least 33% Ala, and 50% Gly; and wherein,

$n=2, 4, 8, 16, 32, 64, 128$ ;

$q=0, 1, 2, 4, 8, 16, 32, 64, 128$ ;

$p=2, 4, 8, 16, 32, 64, 128$ ;

$i=1-128$ ; and

where  $p \geq n$  or  $q$ .

17. The green plant of Claim 16 wherein the silk-like protein has the general formula selected from the group consisting of:  $[(A)_4 - (X)_8]_8$ ,

$[(A)_4 - (X)_8 - (S)]_8$ ,  $[(A)_4 - (X)_8 - (E)]_8$ ,  $[(A)_8 - (X)_8]_8$ ,  $[(A)_4 - (S) - (X)_8]_8$ ,

$[(A)_4 - (S)_2 - (X)_8]_8$ ,  $[(A)_4 - (E) - (X)_8 - (E)]_8$ ,  $[(A)_4 - (E) - (X)_8]_8$ ,  $[(A)_4 - (S) - (X)_8 - (E)]_8$ , and  $[(A)_4 - (S)_2 - (X)_8 - (E)]_8$ .

18. The green plant of Claim 17 wherein:

A=SGGAGGAGG;

E=GPGQQGPGGY;

S=GAGAGY; and

X=SGAGAG.

19. The green plant of Claim 18 wherein the silk-like protein is a spider silk variant having the general formula:

[ACGQGGYGGLGXQGAGRGLGGQGAGAnGG]<sub>z</sub>

wherein X=S, G or N; n=0-7 and z=1-75, and wherein the value of z determines the number of repeats in the variant protein and wherein the formula encompasses variations selected from the group consisting of:

- 5                   (a) when n=0 the sequence encompassing  
AGRGLGGQGAGAnGG is deleted;
- (b) deletions other than the poly-alanine sequence, limited by the  
value of n will encompass integral multiples of three consecutive  
residues;
- 10               (c) the deletion of GYG in any repeat is accompanied by deletion of  
GRG in the same repeat; and
- (d) where a first repeat where n=0 is deleted, the first repeat is  
preceded by a second repeat where n=6; and

wherein the full-length protein is encoded by a gene or genes and wherein said  
15 gene or genes are not endogenous to the *Nephila clavipes* genome.

20. The green plant of Claim 16 selected from the group consisting of  
monocots and dicots.

21. The green plant of Claim 16 selected from the group consisting of  
soybean, rapeseed, sunflower, cotton, corn, tobacco, alfalfa, wheat, barley, oats,  
20 sorghum, rice, *Arabidopsis*, sugar beet, sugar cane, canola, millet, beans, peas,  
rye, flax, grasses, and banna.

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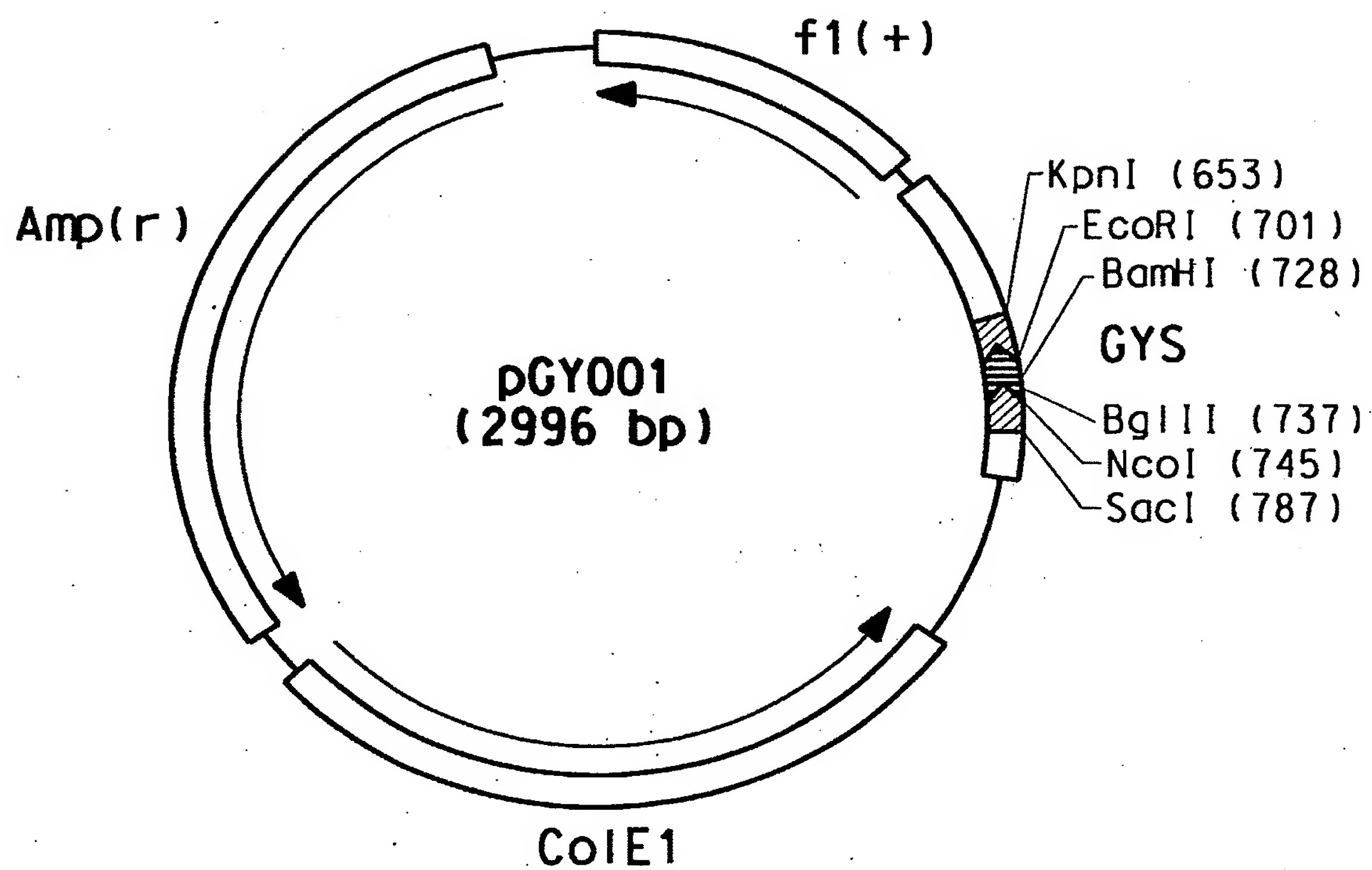


FIG. 1

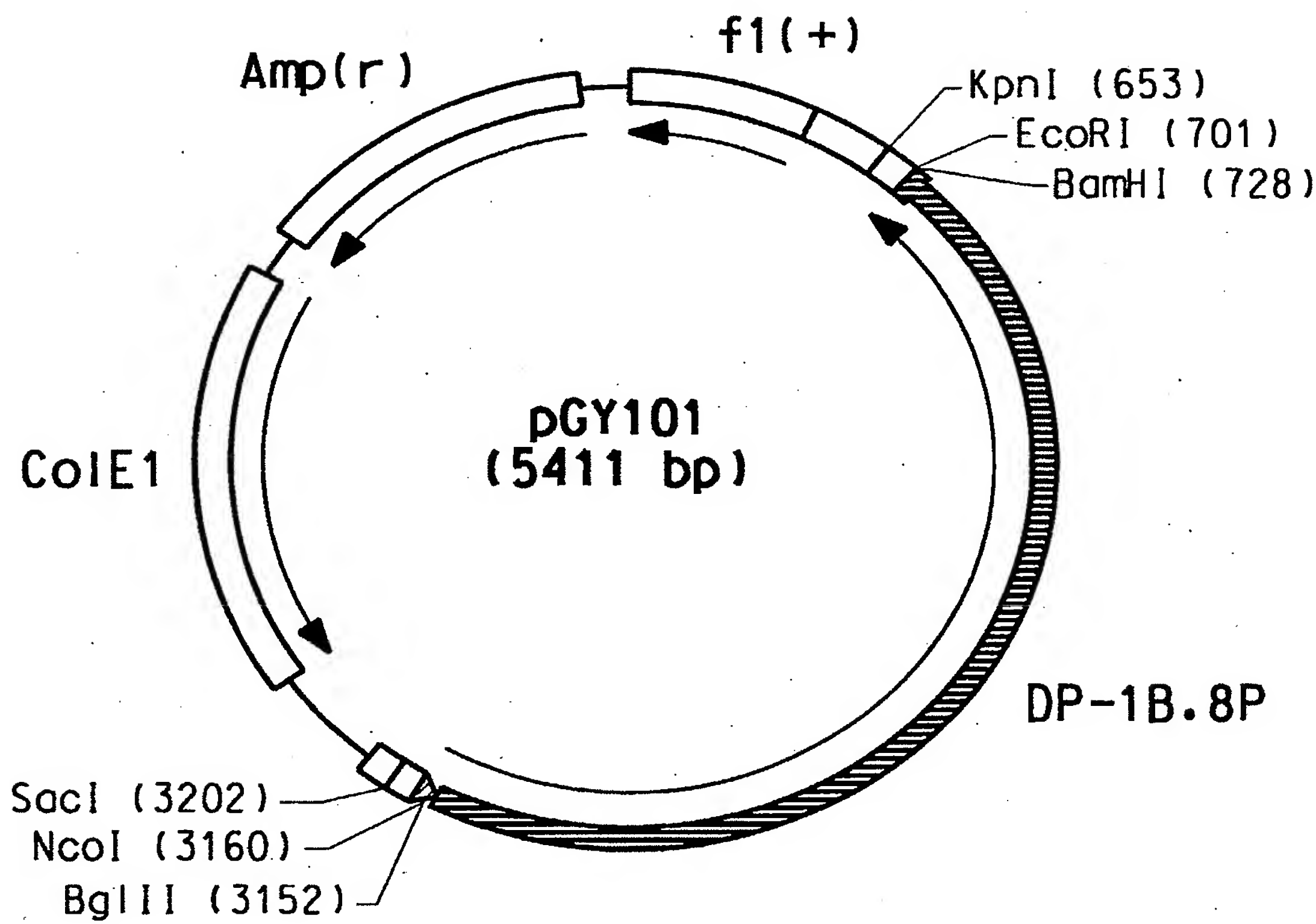


FIG. 2A



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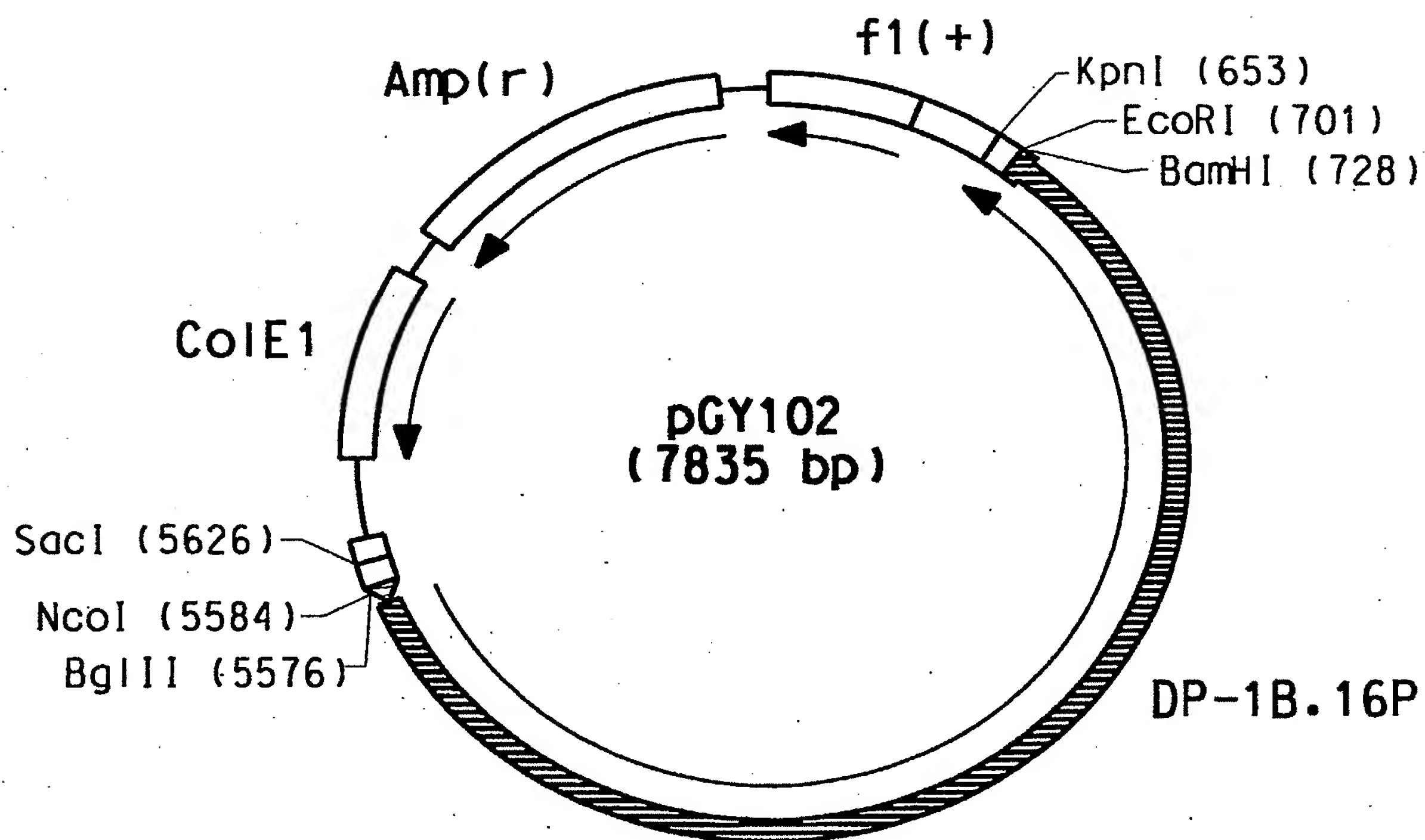


FIG. 2B

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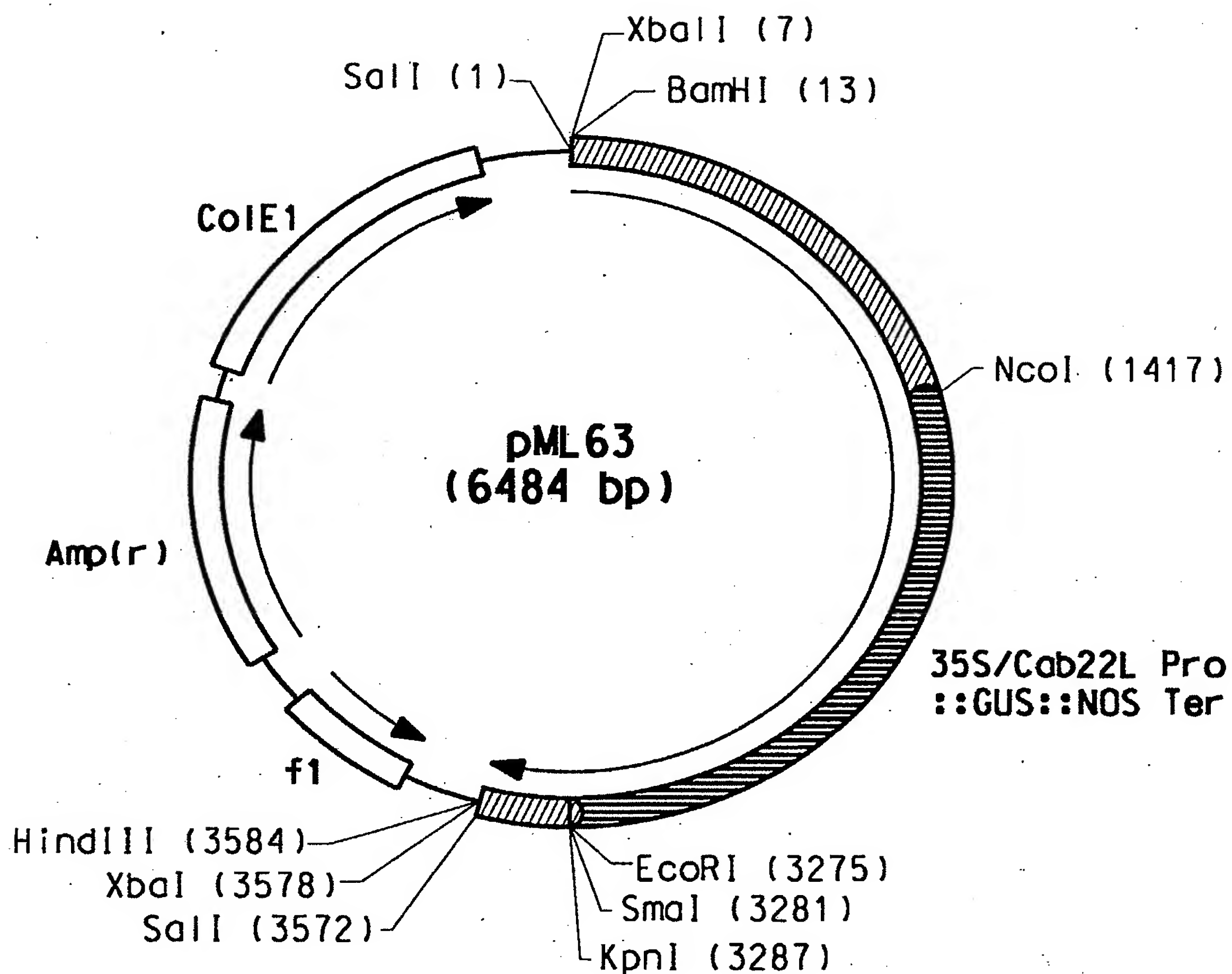


FIG. 3A

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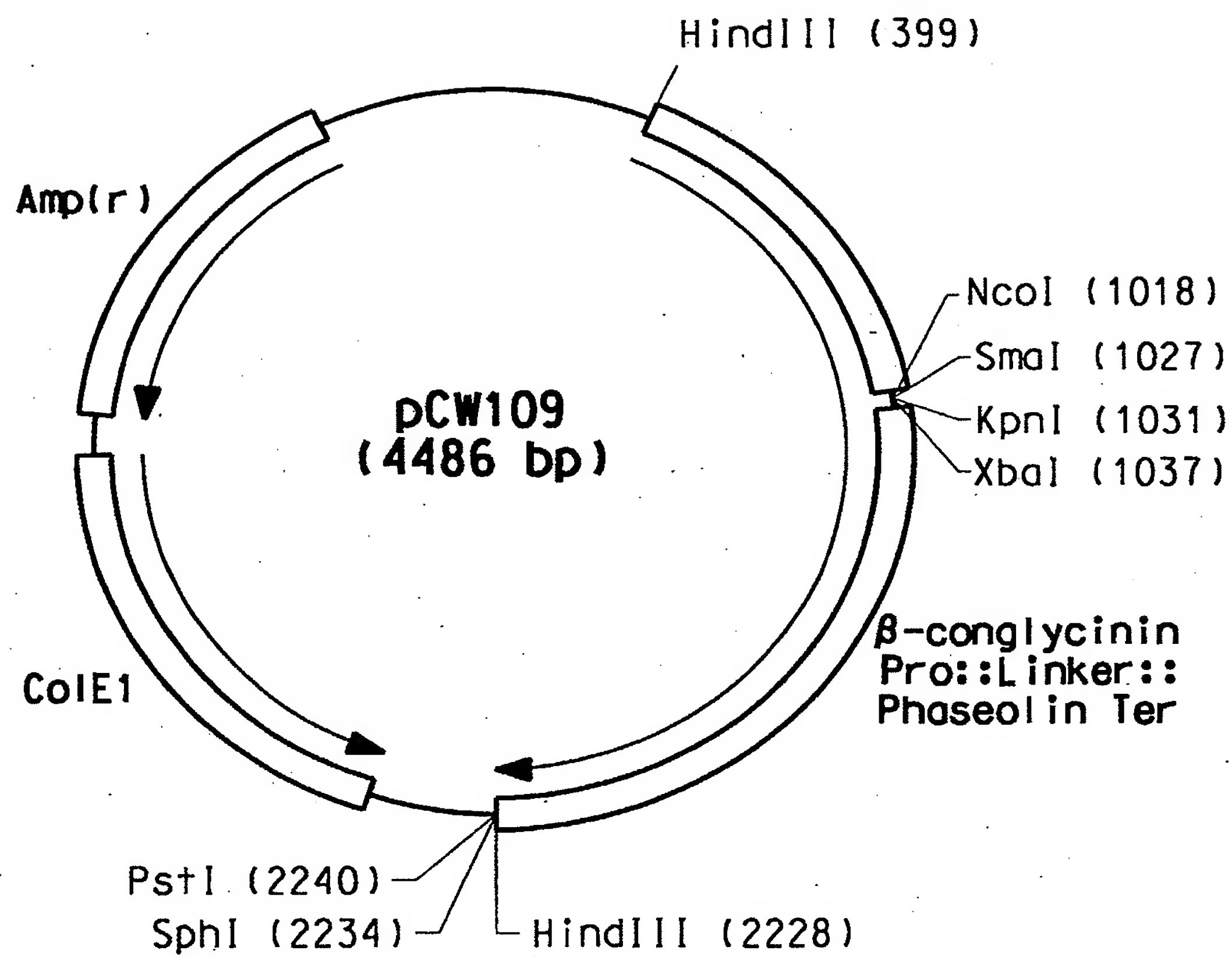


FIG. 3B

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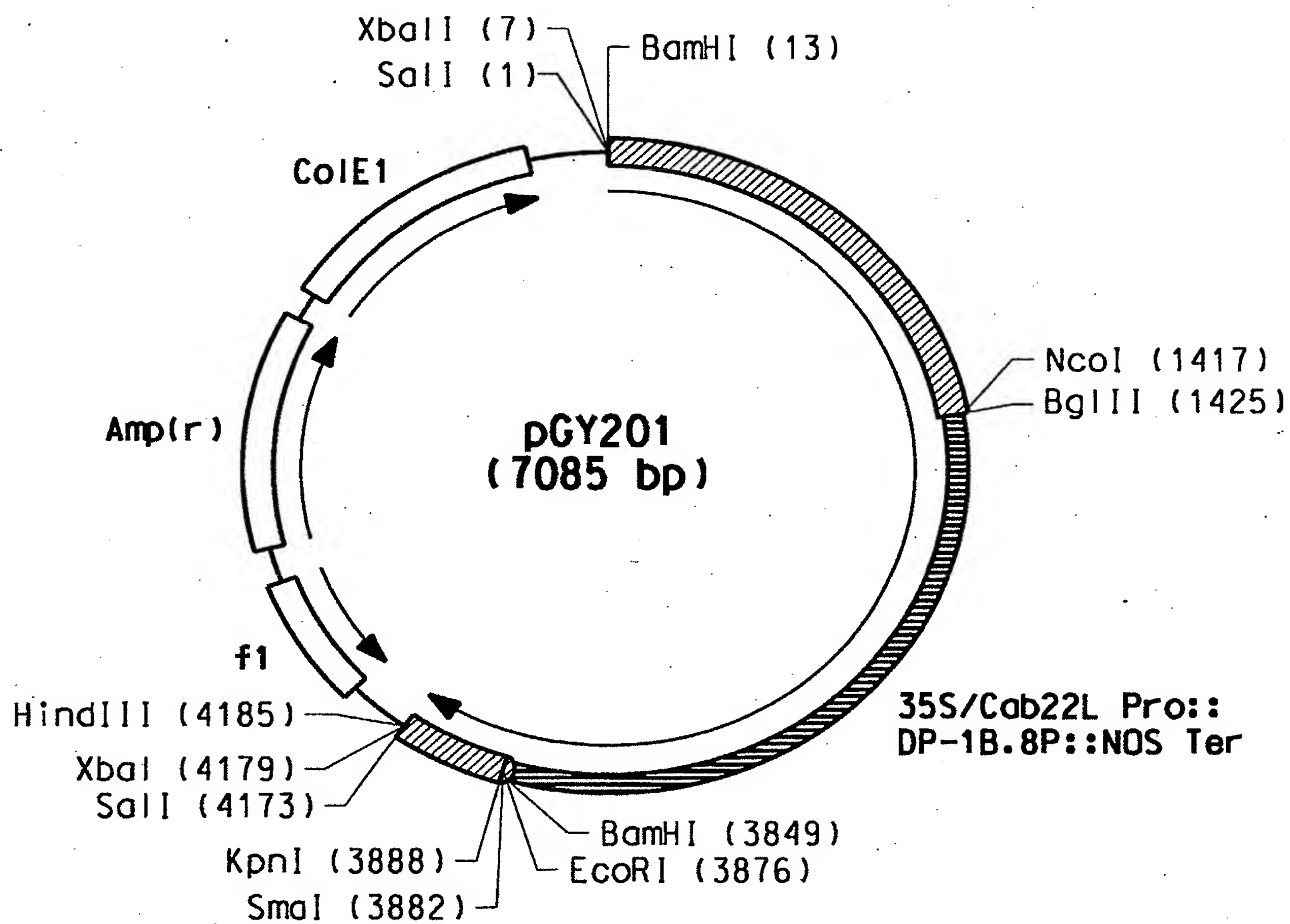


FIG. 4A



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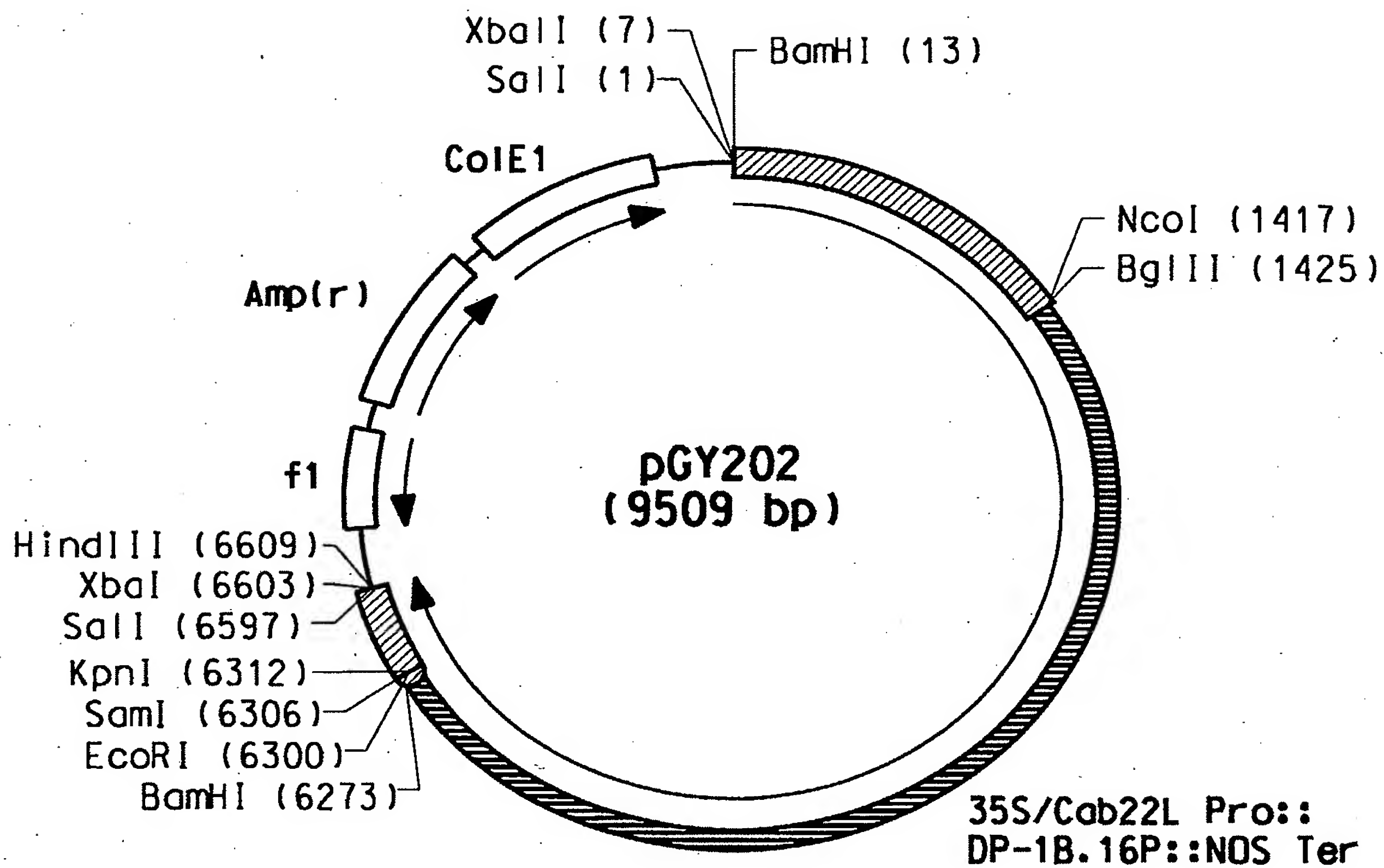


FIG. 4B

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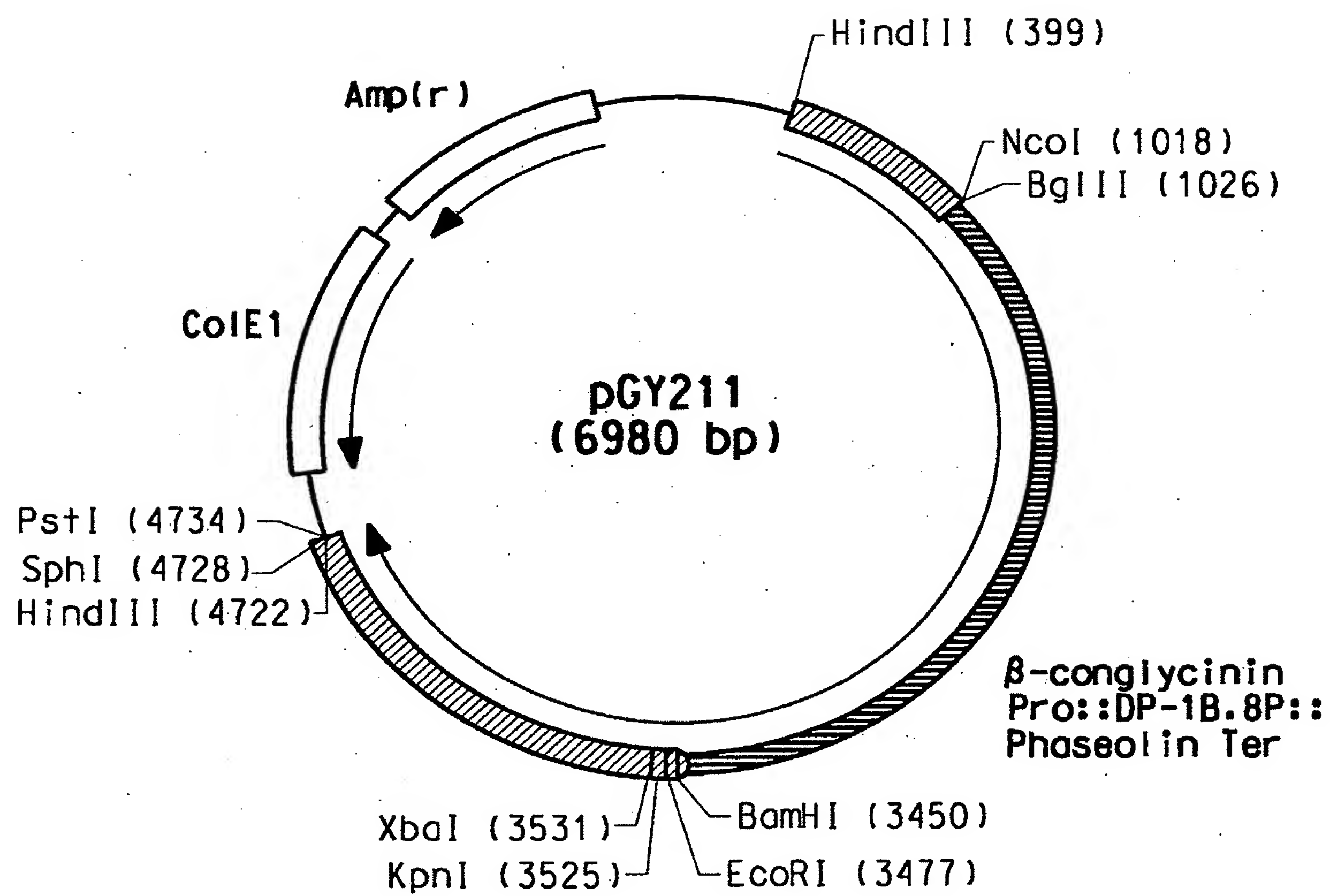


FIG. 5A

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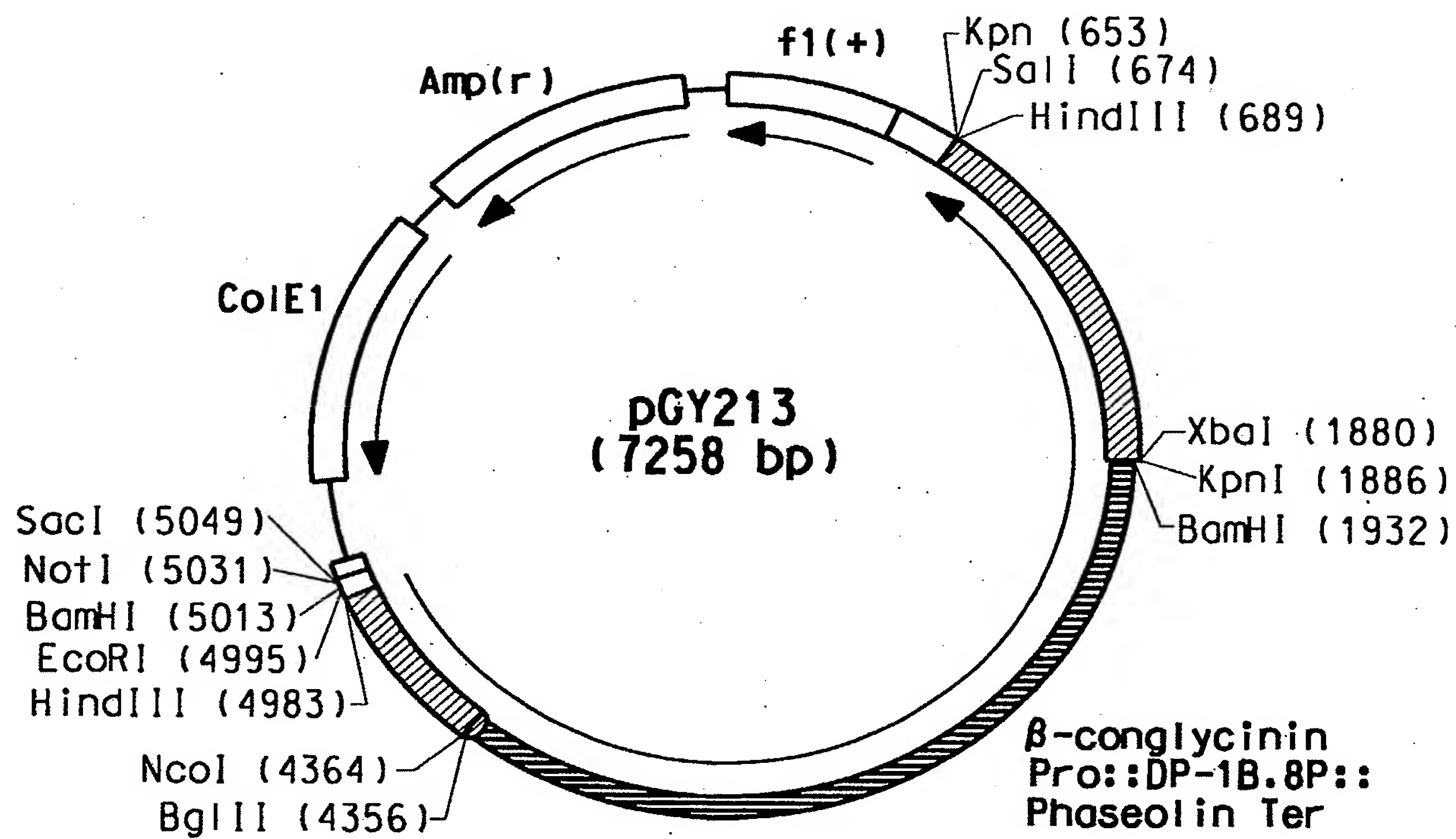


FIG. 5B

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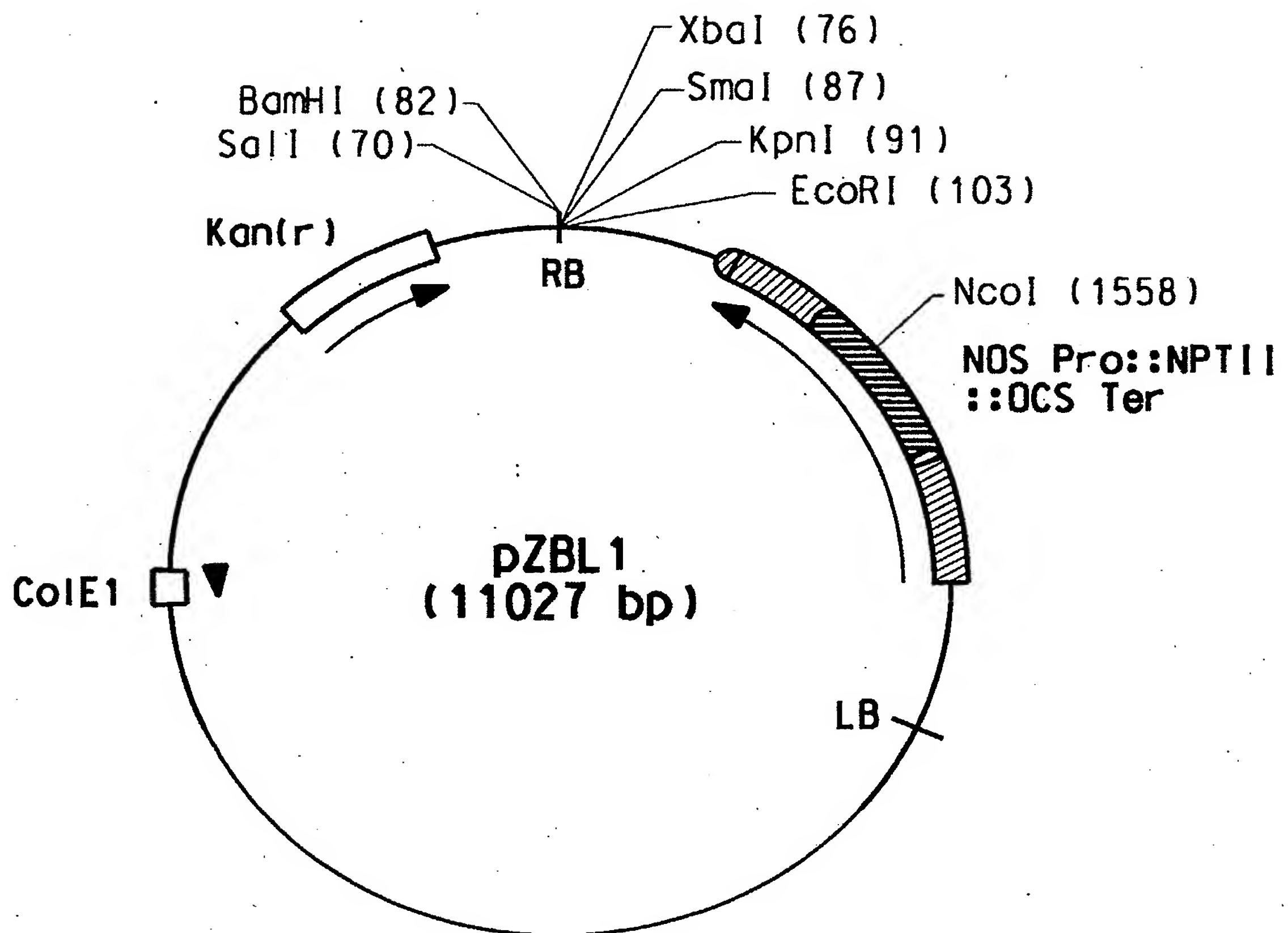


FIG. 6



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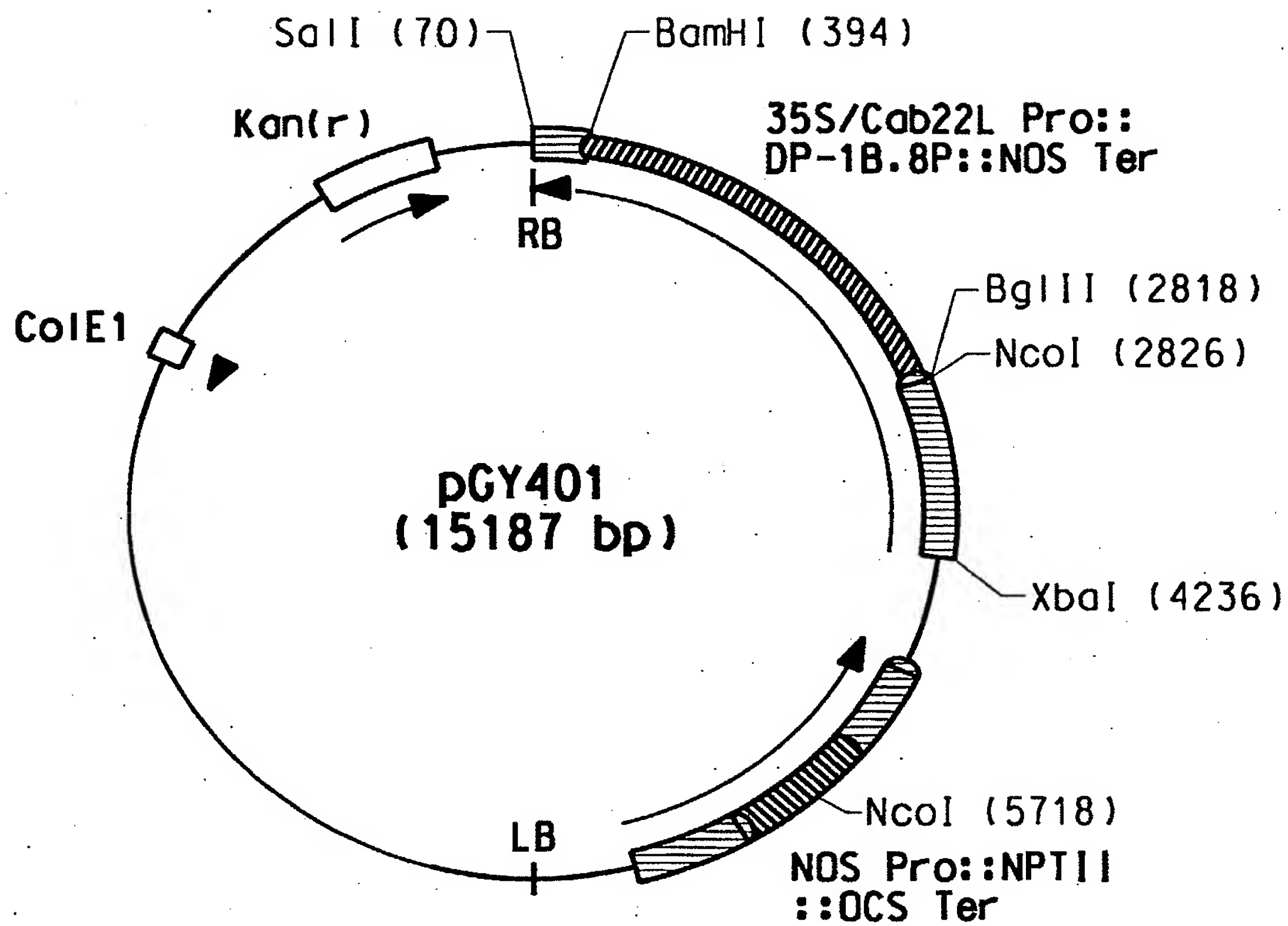


FIG. 7A

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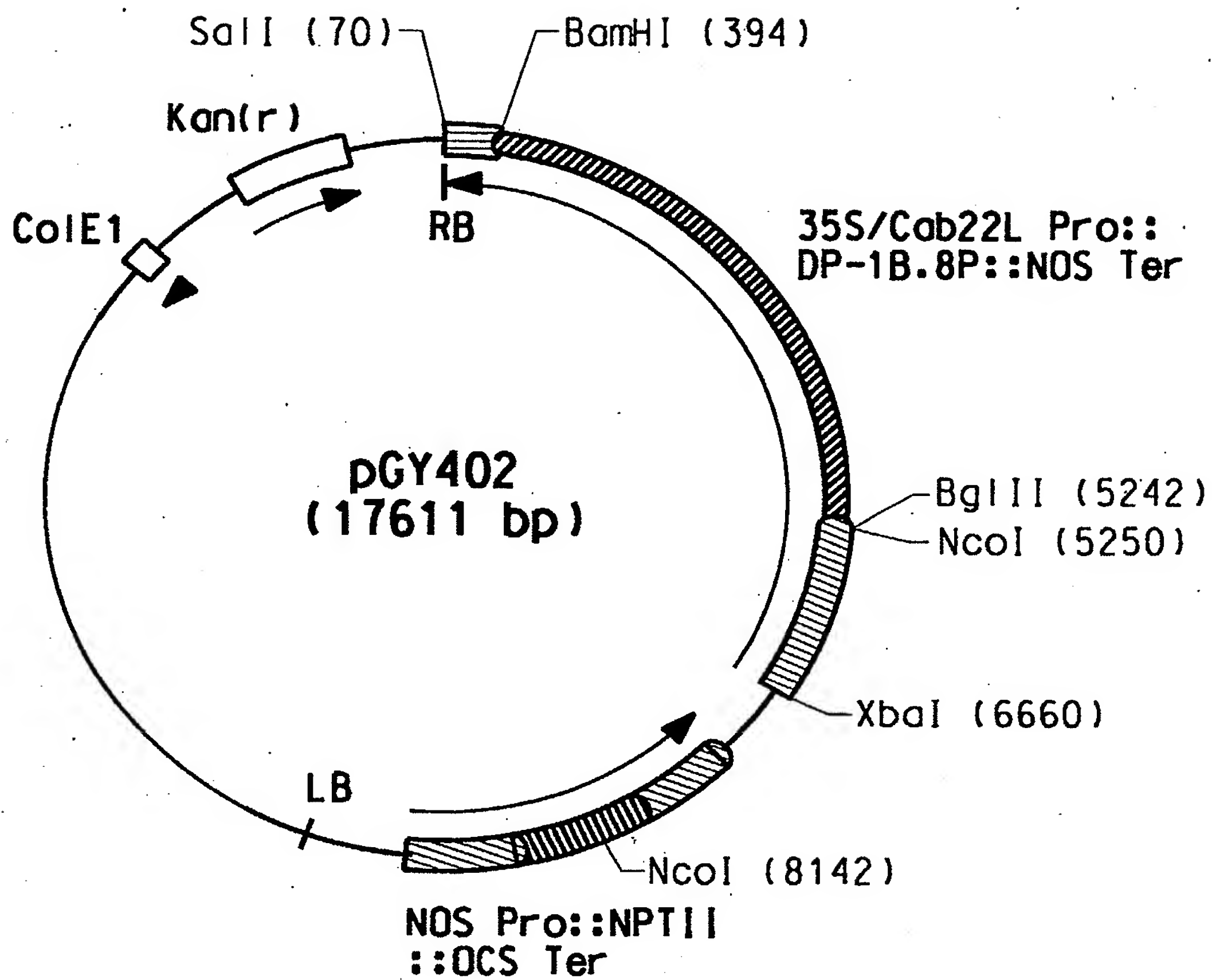


FIG. 7B

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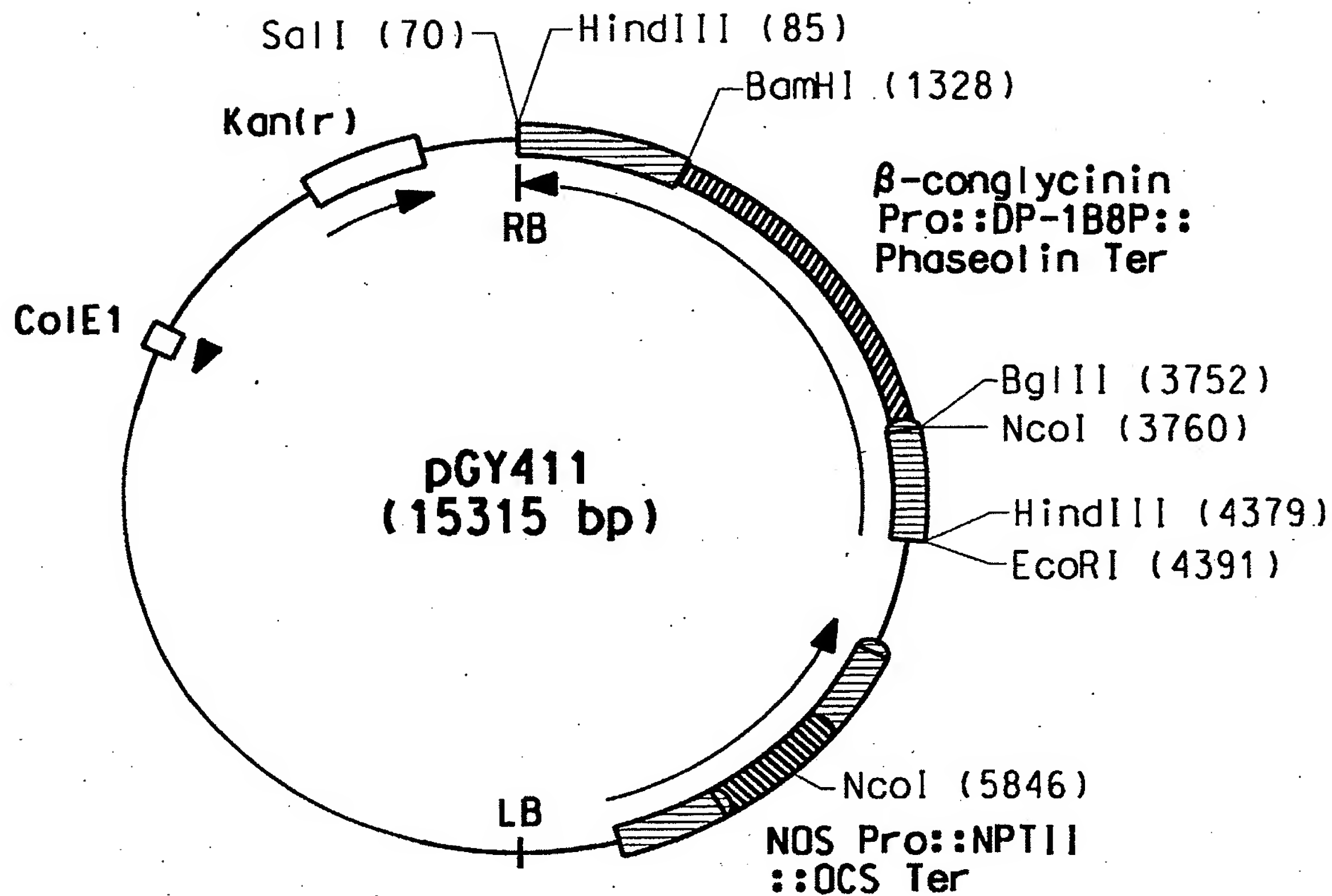


FIG. 8A

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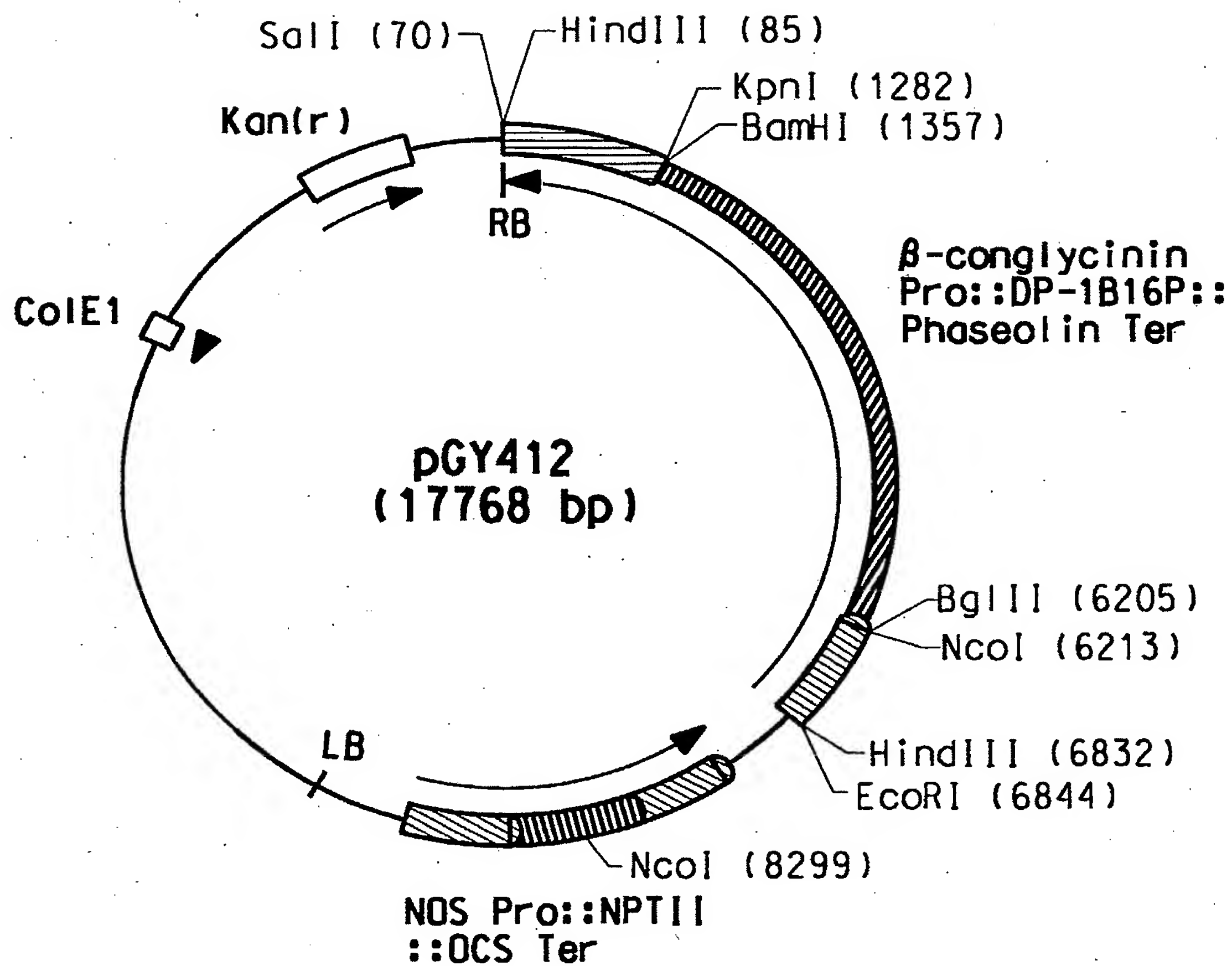


FIG. 8B



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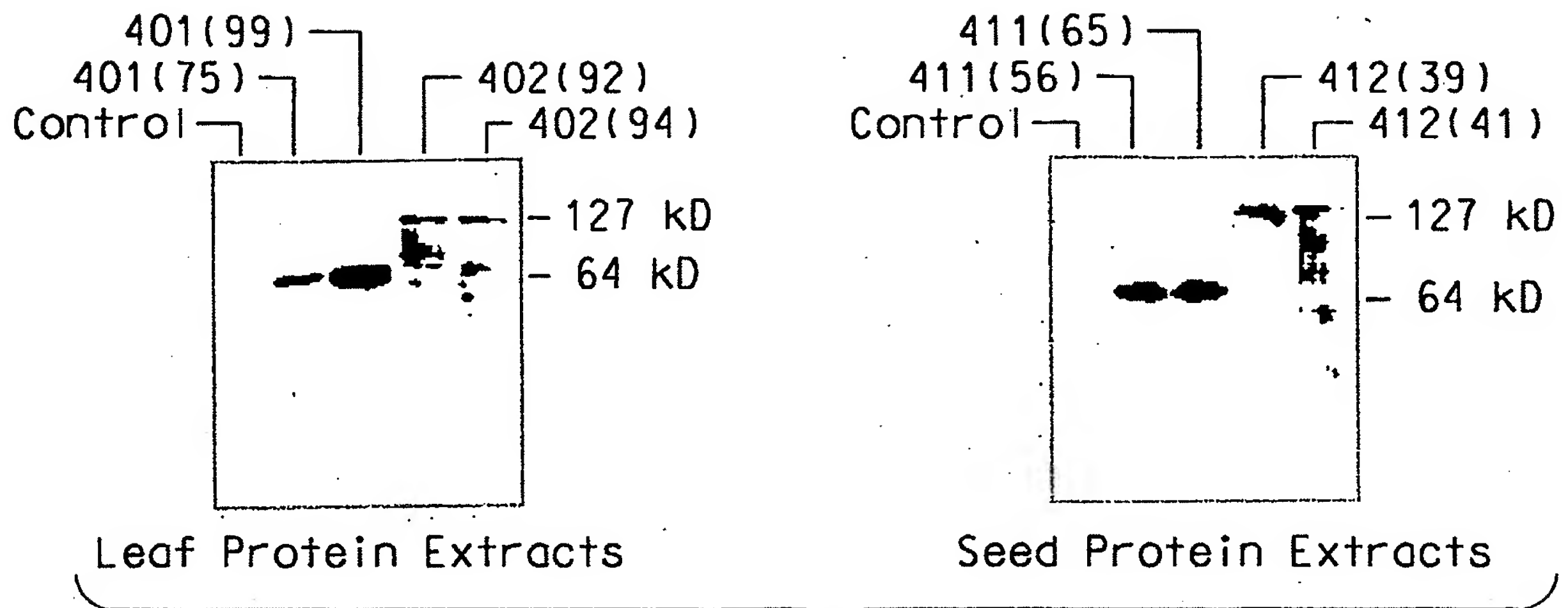


FIG. 9A

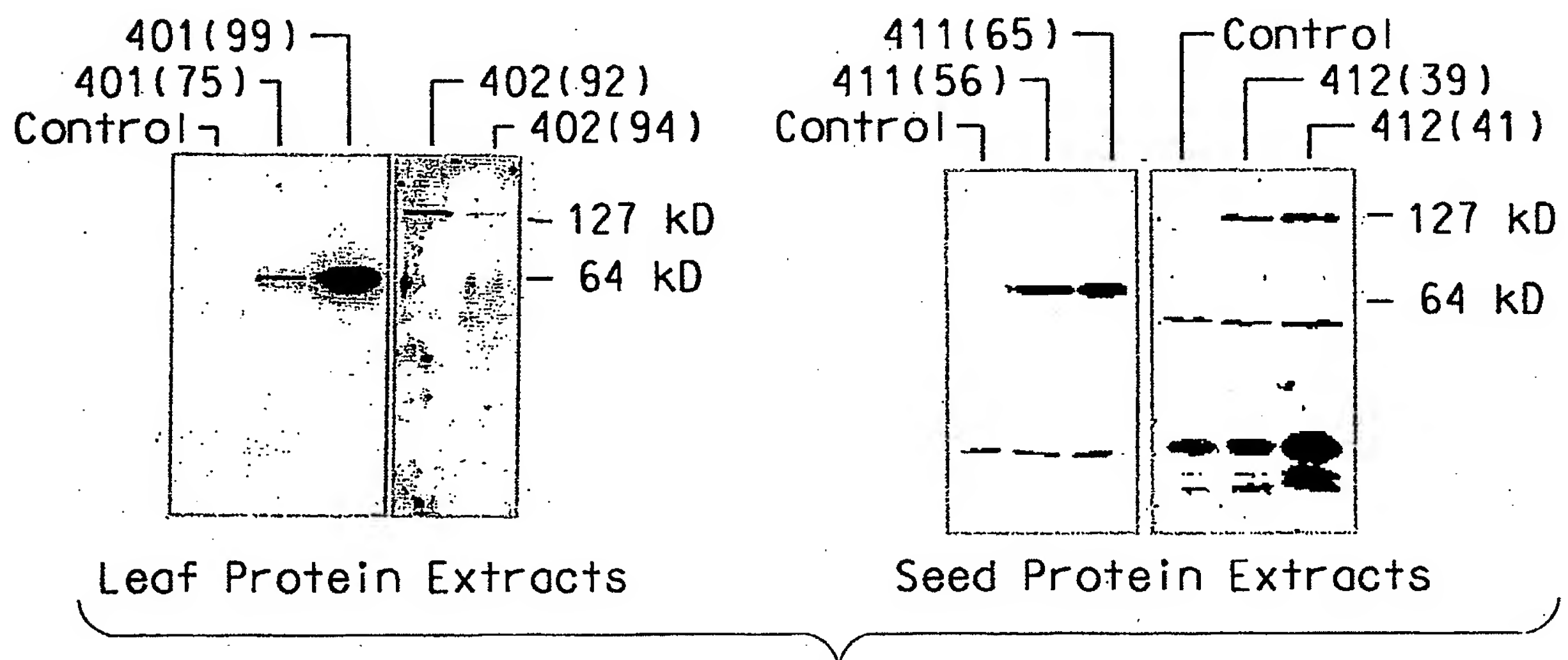


FIG. 9B

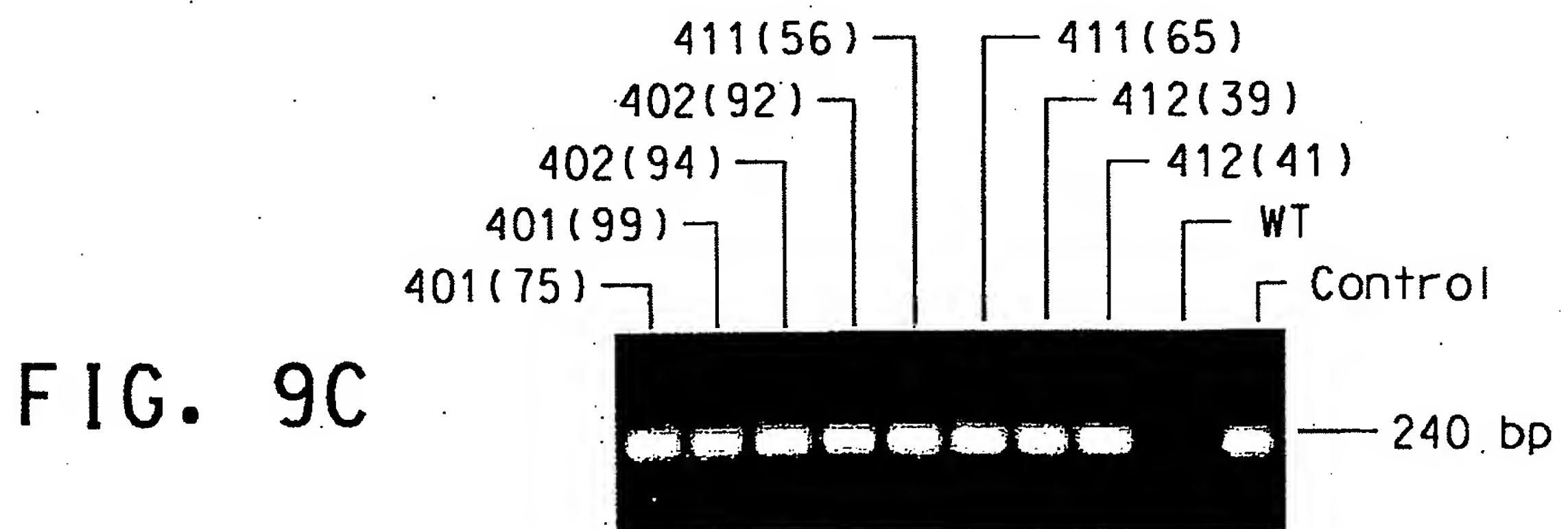


FIG. 9C

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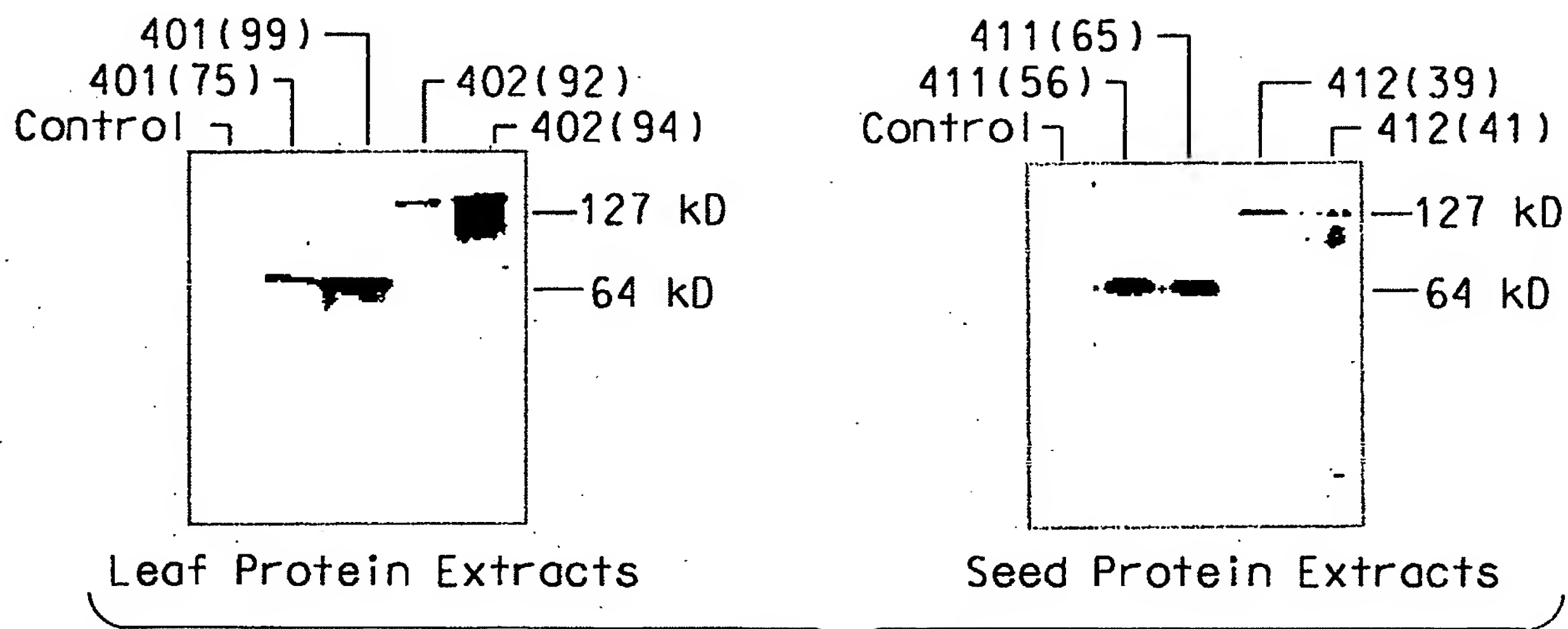


FIG. 10A

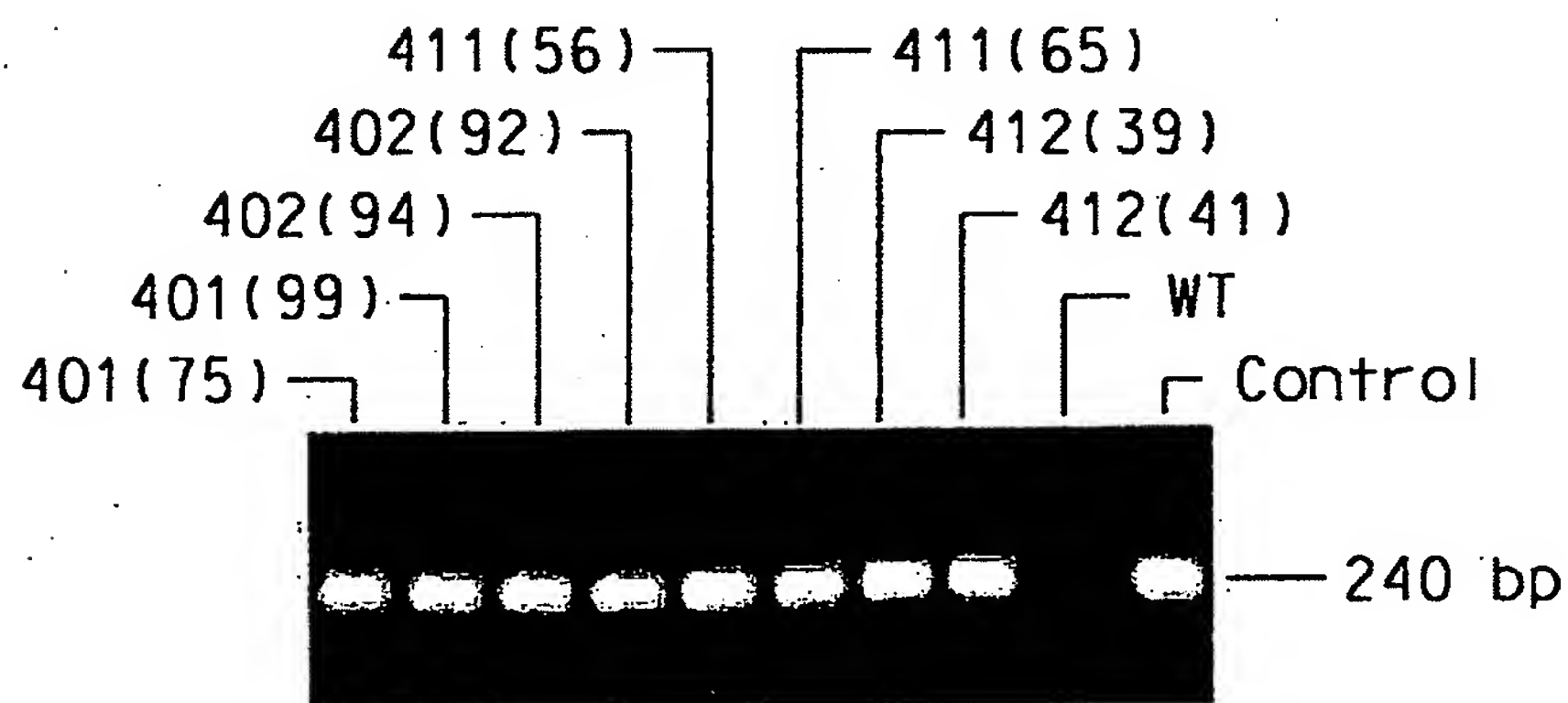


FIG. 10B

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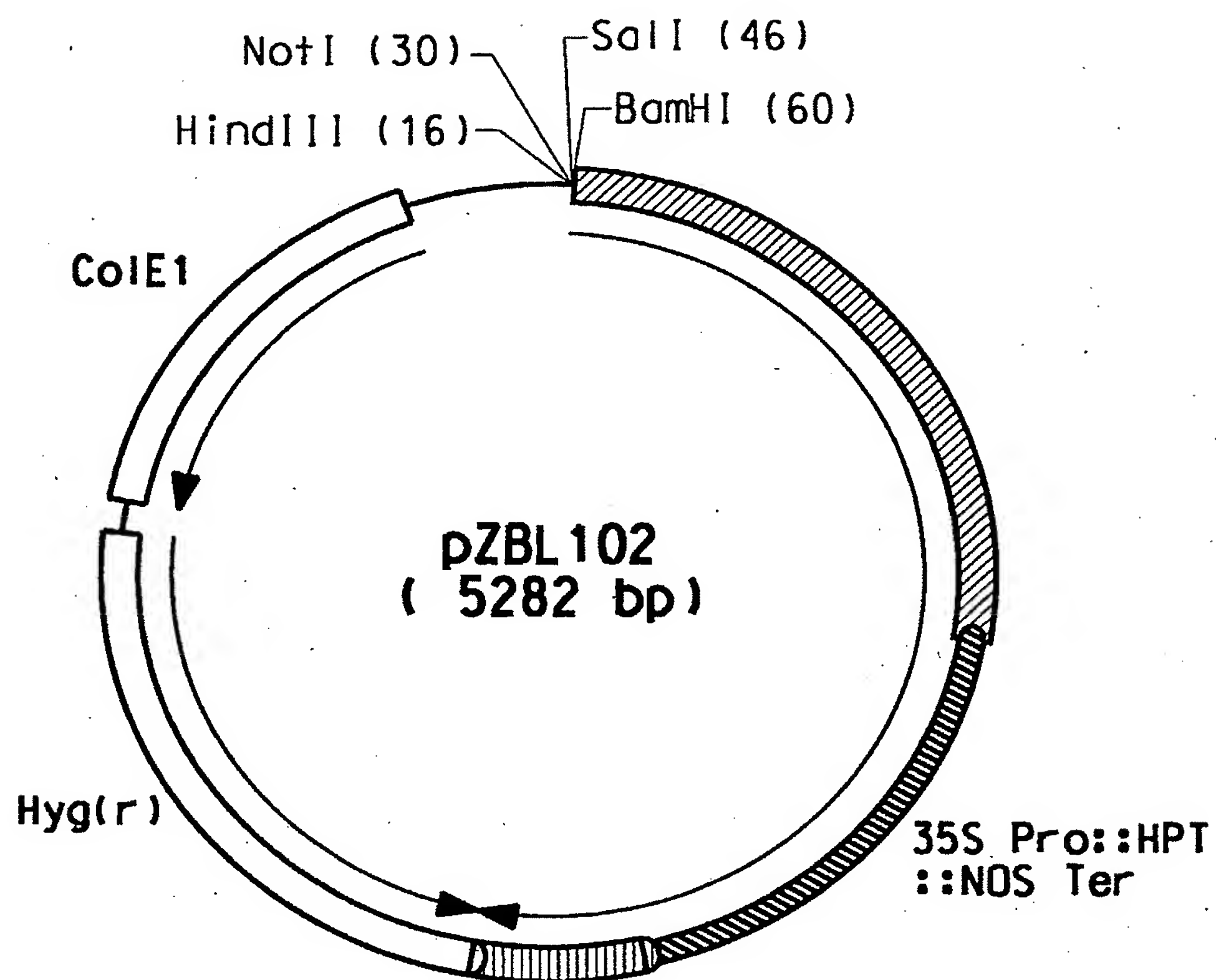


FIG. 11A

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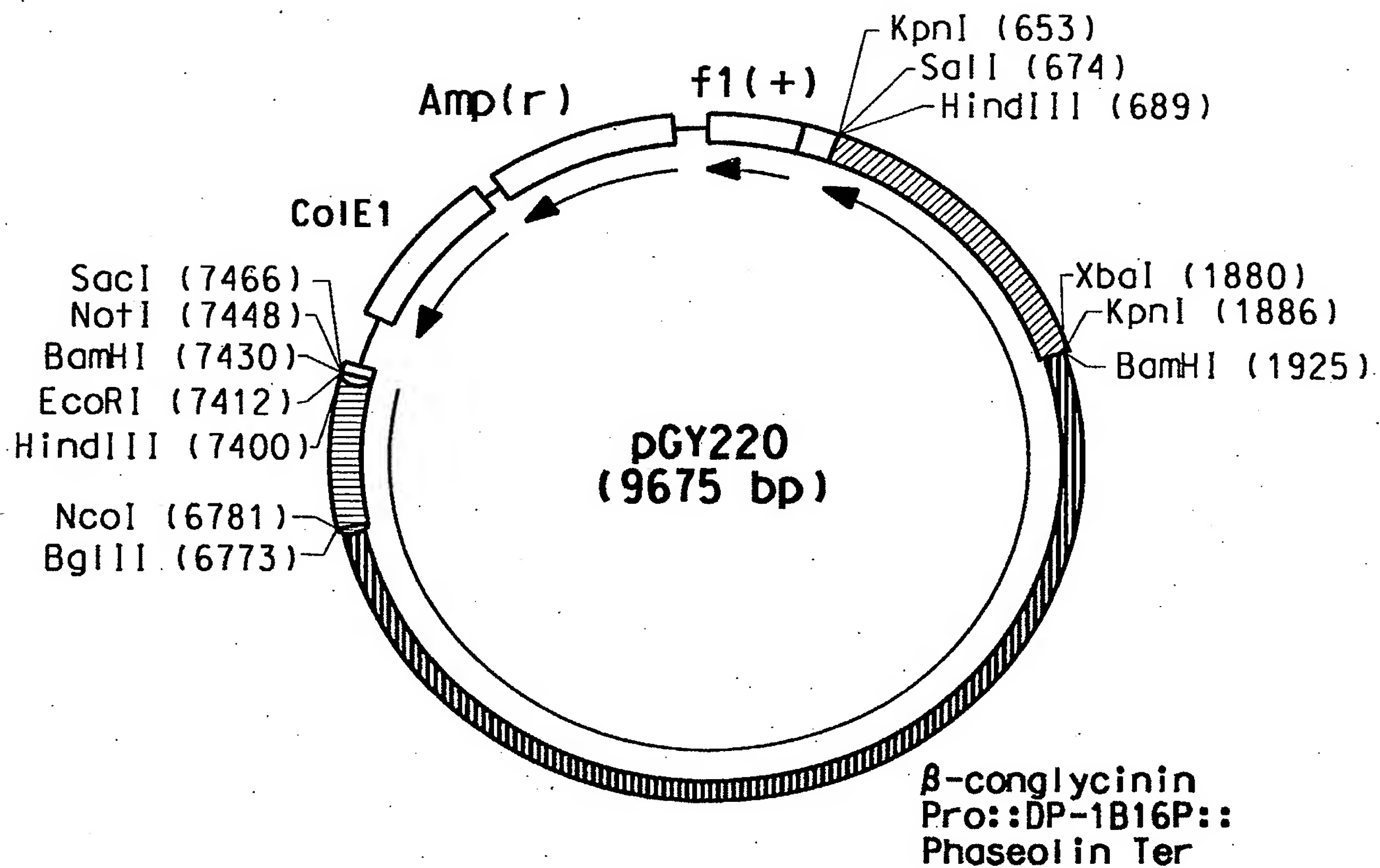


FIG. 11B



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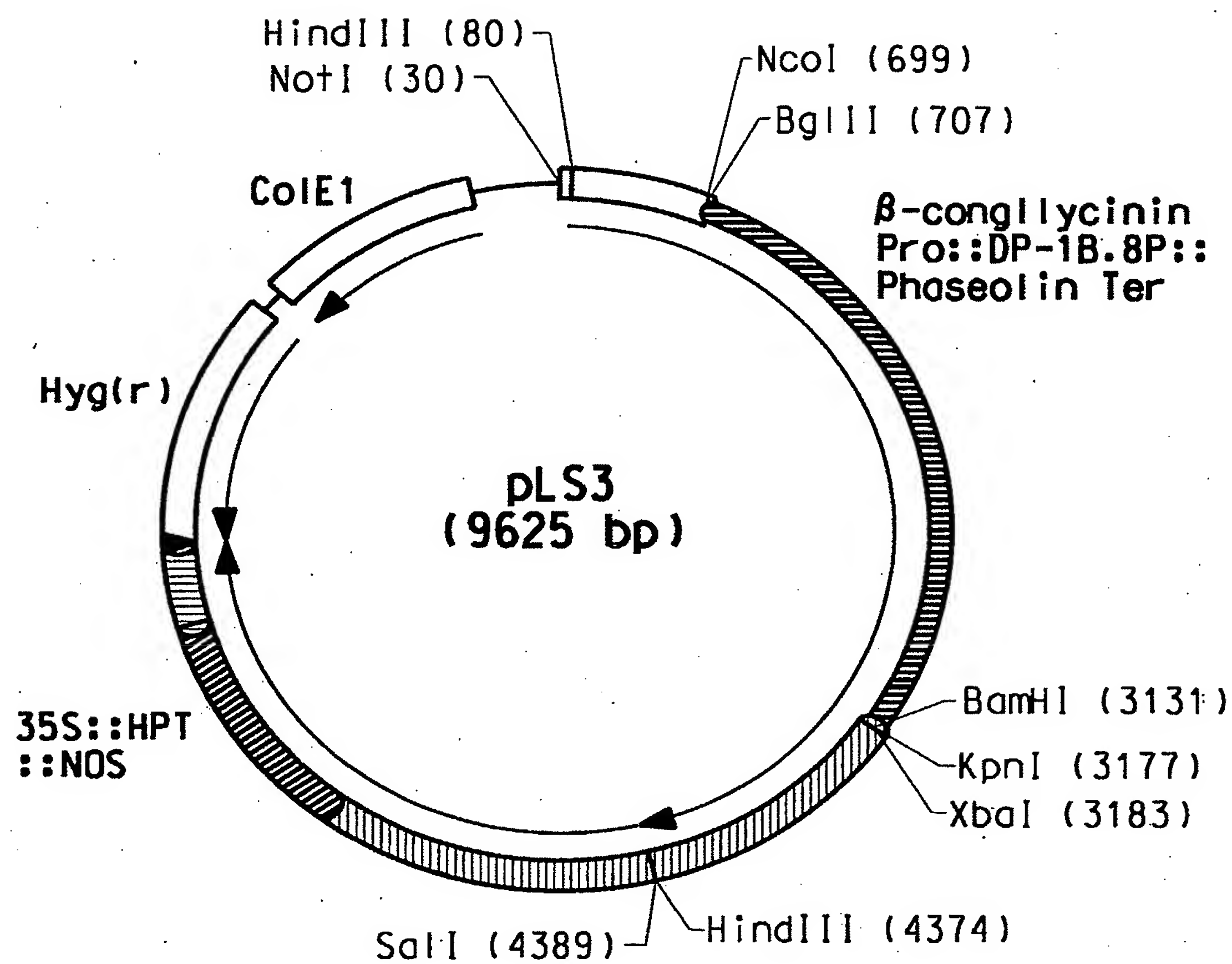


FIG. 12A

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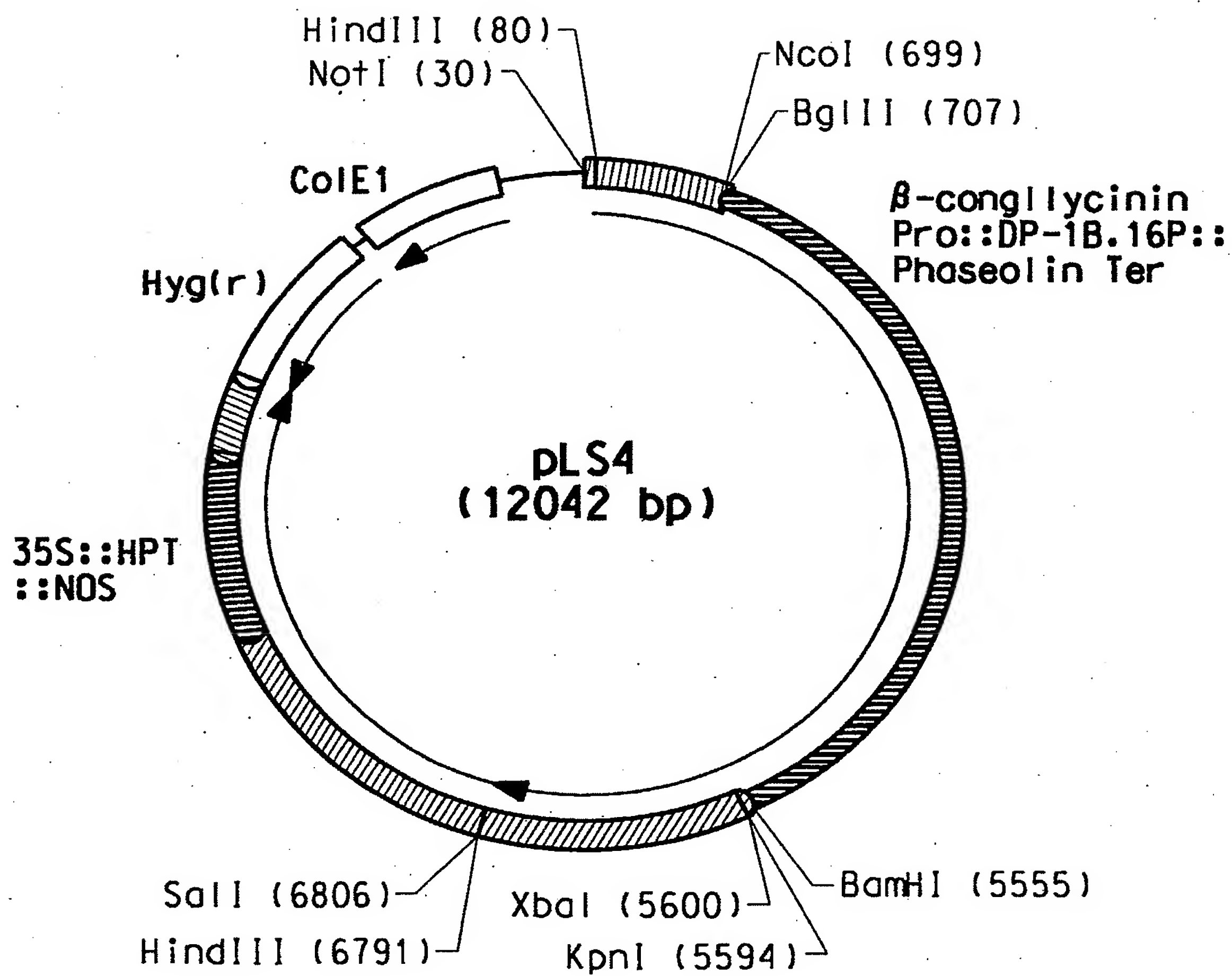


FIG. 12B

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FIG. 13A

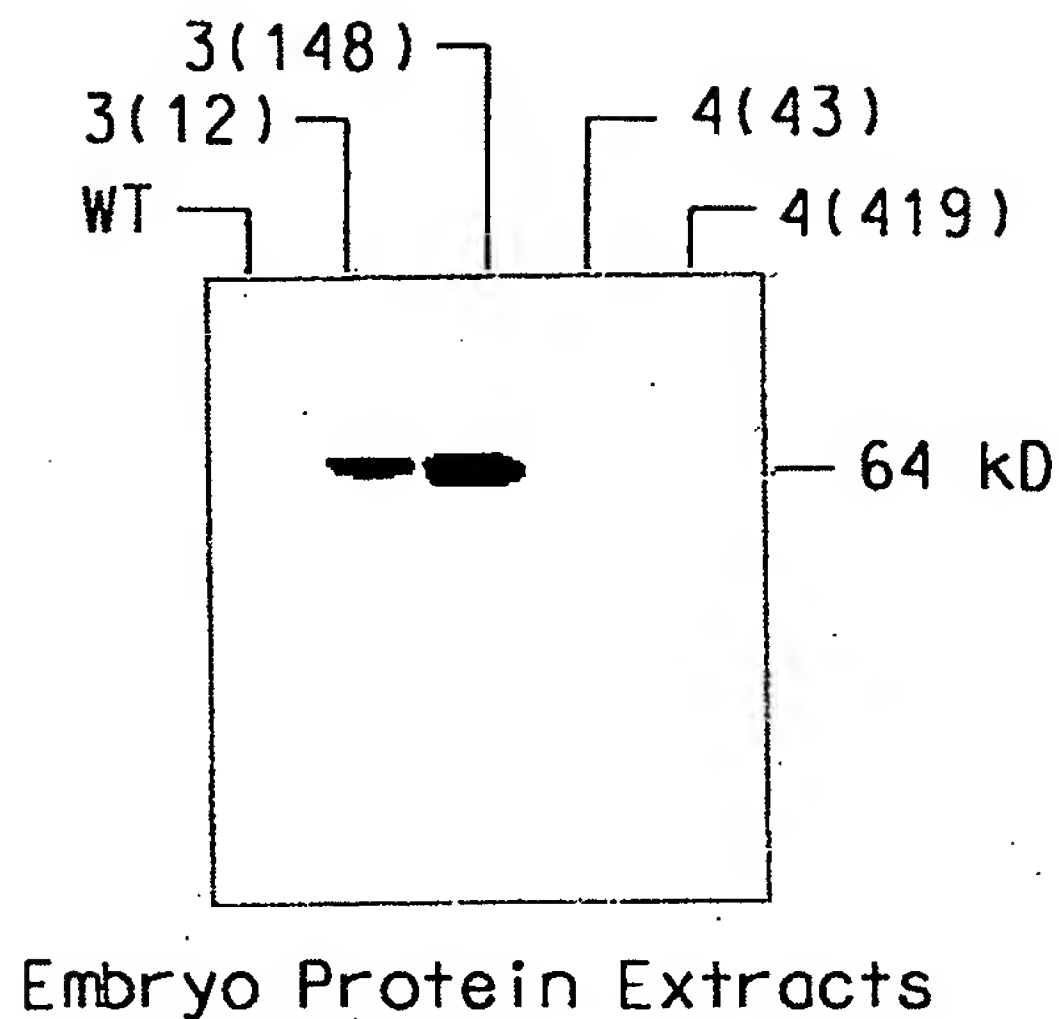


FIG. 13B

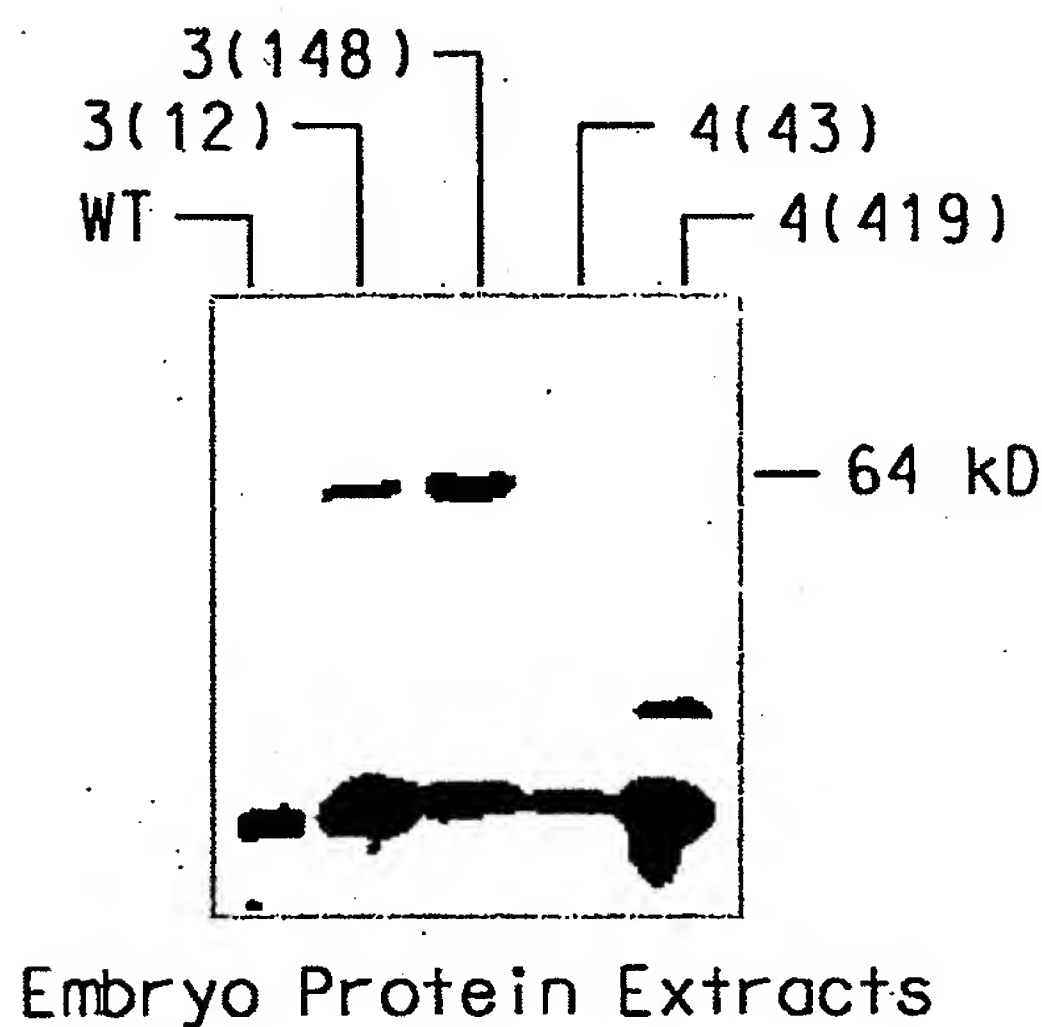
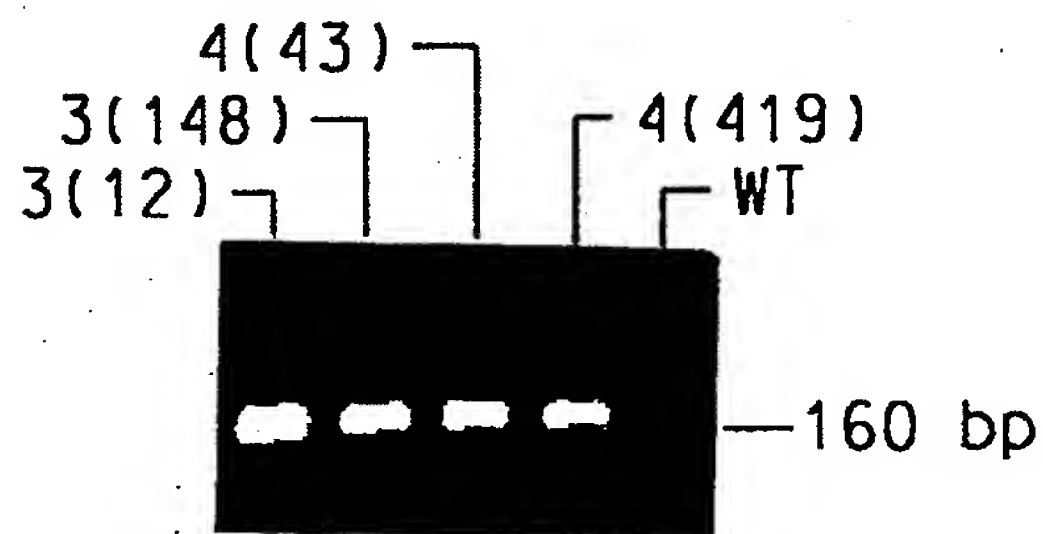


FIG. 13C



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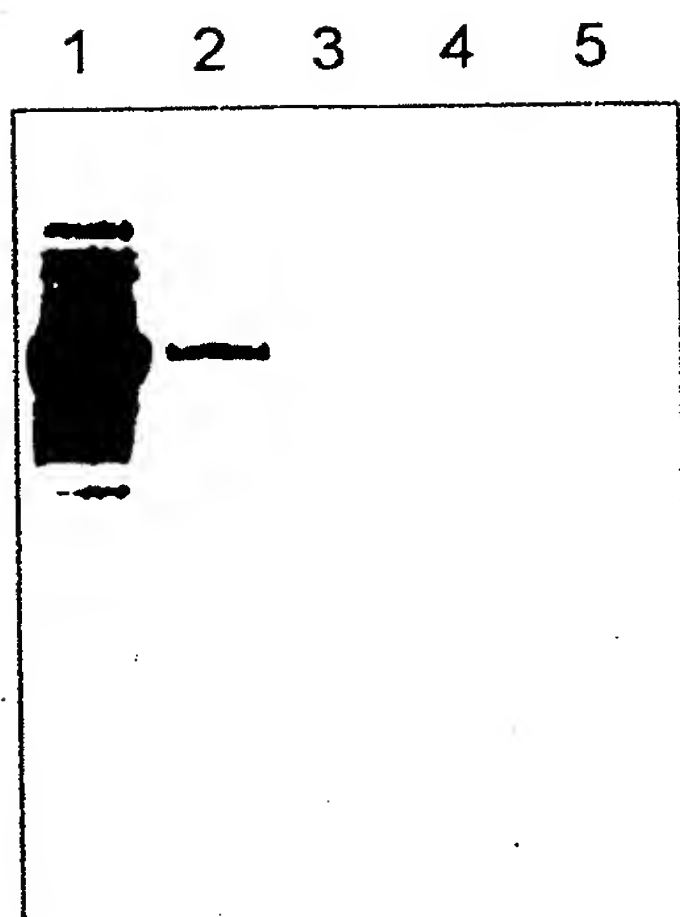


FIG. 14A

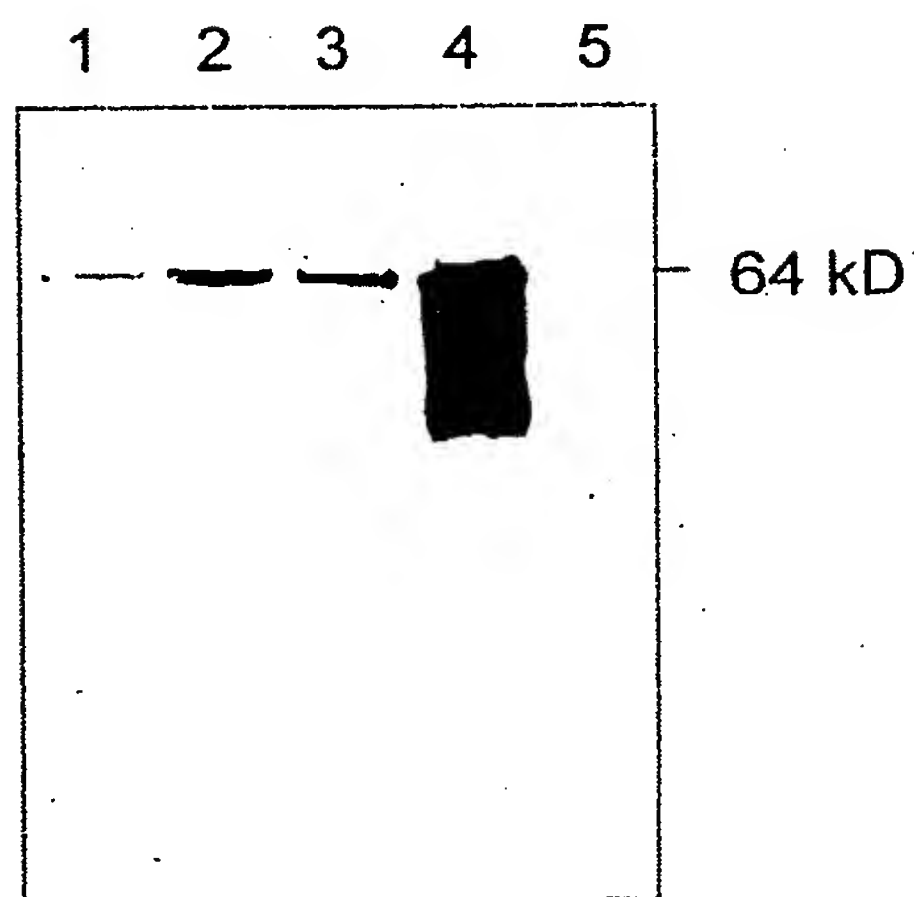


FIG. 14B



SEQUENCE LISTING

&lt;110&gt; E.I. du Pont de Nemours and Company

&lt;120&gt; Production of Silk-Like Proteins in Plants

&lt;130&gt; BC1014 PCT

&lt;140&gt;

&lt;141&gt;

&lt;150&gt; 60/206968

&lt;151&gt; MAY 25, 2000

&lt;160&gt; 29

&lt;170&gt; Microsoft Office 97

&lt;210&gt; 1

&lt;211&gt; 651

&lt;212&gt; PRT

&lt;213&gt; Nephila clavipes

&lt;400&gt; 1

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 20 25 30

Gly Leu Gly Gly Gln Gly Ala Gly Gln Gly Ala Gly Ala Ala Ala Ala  
 35 40 45

Ala Ala Ala Gly Gly Ala Gly Gln Gly Gly Tyr Gly Gly Leu Gly Ser  
 50 55 60

Gln Gly Ala Gly Arg Gly Gly Gln Gly Ala Gly Ala Ala Ala Ala Ala  
 65 70 75 80

Ala Gly Gly Ala Gly Gln Gly Gly Tyr Gly Gly Leu Gly Ser Gln Gly  
 85 90 95

Ala Gly Arg Gly Gly Leu Gly Gly Gln Gly Ala Gly Ala Ala Ala Ala  
 100 105 110

Ala Ala Ala Gly Gly Ala Gly Gln Gly Gly Tyr Gly Gly Leu Gly Asn  
 115 120 125

Gln Gly Ala Gly Arg Gly Gly Gln Gly Ala Ala Ala Ala Ala Ala Gly  
 130 135 140

Gly Ala Gly Gln Gly Gly Tyr Gly Gly Leu Gly Ser Gln Gly Ala Gly  
 145 150 155 160

Arg Gly Gly Leu Gly Gly Gln Gly Ala Gly Ala Ala Ala Ala Ala Ala  
 165 170 175

Gly Gly Ala Gly Gln Gly Gly Tyr Gly Gly Leu Gly Gly Gln Gly Ala  
 180 185 190

Gly Gln Gly Gly Tyr Gly Gly Leu Gly Ser Gln Gly Ala Gly Arg Gly  
 195 200 205

Gly Leu Gly Gly Gln Gly Ala Gly Ala Ala Ala Ala Ala Ala Gly  
 210 215 220  
 Gly Ala Gly Gln Gly Gly Leu Gly Gly Gln Gly Ala Gly Gln Gly Ala  
 225 230 235 240  
 Gly Ala Ser Ala Ala Ala Ala Gly Gly Ala Gly Gln Gly Gly Tyr Gly  
 245 250 255  
 Gly Leu Gly Ser Gln Gly Ala Gly Arg Gly Gly Glu Gly Ala Gly Ala  
 260 265 270  
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 275 280 285  
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 290 295 300  
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 305 310 315 320  
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 340 345 350  
 Tyr Gly Gly Leu Gly Ser Gln Gly Ala Gly Arg Gly Gly Leu Gly Gly  
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 Gln Gly Ala Gly Ala Val Ala Ala Ala Ala Gly Gly Ala Gly Gln  
 370 375 380  
 Gly Gly Tyr Gly Gly Leu Gly Ser Gln Gly Ala Gly Arg Gly Gly Gln  
 385 390 395 400  
 Gly Ala Gly Ala Ala Ala Ala Ala Ala Gly Gly Ala Gly Gln Arg Gly  
 405 410 415  
 Tyr Gly Gly Leu Gly Asn Gln Gly Ala Gly Arg Gly Gly Leu Gly Gly  
 420 425 430  
 Gln Gly Ala Gly Ala Ala Ala Ala Ala Ala Gly Gly Ala Gly Gln  
 435 440 445  
 Gly Gly Tyr Gly Gly Leu Gly Asn Gln Gly Ala Gly Arg Gly Gly Gln  
 450 455 460  
 Gly Ala Ala Ala Ala Ala Gly Gly Ala Gly Gln Gly Gly Tyr Gly Gly  
 465 470 475 480  
 Leu Gly Ser Gln Gly Ala Gly Arg Gly Gly Gln Gly Ala Gly Ala Ala  
 485 490 495  
 Ala Ala Ala Ala Val Gly Ala Gly Gln Glu Gly Ile Arg Gly Gln Gly  
 500 505 510  
 Ala Gly Gln Gly Gly Tyr Gly Gly Leu Gly Ser Gln Gly Ser Gly Arg  
 515 520 525  
 Gly Gly Leu Gly Gly Gln Gly Ala Gly Ala Ala Ala Ala Ala Ala Gly  
 530 535 540

Gly Ala Gly Gln Gly Gly Leu Gly Gly Gln Gly Ala Gly Gln Gly Ala  
 545 550 555 560  
 Gly Ala Ala Ala Ala Ala Gly Gly Val Arg Gln Gly Gly Tyr Gly  
 565 570 575  
 Gly Leu Gly Ser Gln Gly Ala Gly Arg Gly Gly Gln Gly Ala Gly Ala  
 580 585 590  
 Ala Ala Ala Ala Ala Gly Gly Ala Gly Gln Gly Gly Tyr Gly Gly Leu  
 595 600 605  
 Gly Gly Gln Gly Val Gly Arg Gly Gly Leu Gly Gly Gln Gly Ala Gly  
 610 615 620  
 Ala Ala Ala Ala Gly Gly Ala Gly Gln Gly Gly Tyr Gly Gly Val Gly  
 625 630 635 640  
 Ser Gly Ala Ser Ala Ala Ser Ala Ala Ala Ala  
 645 650

<210> 2  
 <211> 6  
 <212> PRT  
 <213> Artificial Sequence

<220>  
 <223> Description of Artificial Sequence: SLP repeat

<400> 2  
 Ser Gly Ala Gly Ala Gly  
 1 5

<210> 3  
 <211> 6  
 <212> PRT  
 <213> Artificial Sequence

<220>  
 <223> Description of Artificial Sequence: SLP repeat

<400> 3  
 Gly Ala Gly Ala Gly Ser  
 1 5

<210> 4  
 <211> 59  
 <212> PRT  
 <213> Artificial Sequence

<220>  
 <223> Description of Artificial Sequence: SLP repeat

<400> 4  
 Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala  
 1 5 10 15  
 Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala  
 20 25 30  
 Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser  
 35 40 45

Gly Ala Gly Ala Gly Ser Gly Ala Ala Gly Tyr  
           50                                  55

<210> 5  
 <211> 9  
 <212> PRT  
 <213> Artificial Sequence

<220>  
 <223> Description of Artificial Sequence: SLP repeat

<400> 5  
 Ser Gly Gly Ala Gly Gly Ala Gly Gly  
       1                                  5

<210> 6  
 <211> 10  
 <212> PRT  
 <213> Artificial Sequence

<220>  
 <223> Description of Artificial Sequence: SLP repeat

<400> 6  
 Gly Pro Gly Gln Gln Gly Pro Gly Gly Tyr  
       1                                  5                                  10

<210> 7  
 <211> 6  
 <212> PRT  
 <213> Artificial Sequence

<220>  
 <223> Description of Artificial Sequence: SLP repeat

<400> 7  
 Gly Ala Gly Ala Gly Tyr  
       1                                  5

<210> 8  
 <211> 34  
 <212> PRT  
 <213> Artificial Sequence

<220>  
 <223> Description of Artificial Sequence: SLP repeat

<220>  
 <221> UNSURE  
 <222> (11)  
 <223> X=S, G OR N

<400> 8  
 Ala Gly Gln Gly Gly Tyr Gly Gly Leu Gly Xaa Gln Gly Ala Gly Arg  
       1                                  5                                  10                                  15

Gly Gly Leu Gly Gly Gln Gly Ala Gly Ala Ala Ala Ala Ala Ala  
                                   20                                  25                                  30

Gly Gly

<210> 9  
 <211> 15

<212> PRT  
 <213> Artificial Sequence

<220>  
 <223> Description of Artificial Sequence: SLP repeat

<400> 9  
 Ala Gly Arg Gly Gly Leu Gly Gly Gln Gly Ala Gly Ala Gly Gly  
 1 5 10 15

<210> 10  
 <211> 101  
 <212> PRT  
 <213> Artificial Sequence

<220>  
 <223> Description of Artificial Sequence: DP-1A monomer

<400> 10  
 Gly Ala Gly Arg Gly Gly Gln Gly Ala Gly Ala Ala Ala Ala Ala Ala  
 1 5 10 15  
 Gly Gly Ala Gly Gln Gly Gly Tyr Gly Gly Leu Gly Ser Gln Gly Ala  
 20 25 30  
 Gly Arg Gly Gly Leu Gly Gly Gln Gly Ala Gly Ala Ala Ala Ala Ala  
 35 40 45  
 Ala Ala Gly Gly Ala Gly Gln Gly Gly Leu Gly Ser Gln Gly Ala Gly  
 50 55 60  
 Gln Gly Ala Gly Ala Ala Ala Ala Ala Gly Gly Ala Gly Gln Gly  
 65 70 75 80  
 Gly Tyr Gly Gly Leu Gly Ser Gln Gly Ala Gly Gln Gly Gly Tyr Gly  
 85 90 95  
 Gly Leu Gly Ser Gln  
 100

<210> 11  
 <211> 101  
 <212> PRT  
 <213> Artificial Sequence

<220>  
 <223> Description of Artificial Sequence: DP-1B monomer

<400> 11  
 Gly Ala Gly Gln Gly Gly Tyr Gly Gly Leu Gly Ser Gln Gly Ala Gly  
 1 5 10 15  
 Arg Gly Gly Leu Gly Gly Gln Gly Ala Gly Ala Ala Ala Ala Ala Ala  
 20 25 30  
 Ala Gly Gly Ala Gly Gln Gly Gly Leu Gly Ser Gln Gly Ala Gly Gln  
 35 40 45  
 Gly Ala Gly Ala Ala Ala Ala Ala Gly Gly Ala Gly Gln Gly Gly  
 50 55 60  
 Tyr Gly Gly Leu Gly Ser Gln Gly Ala Gly Arg Gly Gly Gln Gly Ala  
 65 70 75 80





Ala Gly Gly Ala Gly Gln Gly Gly Tyr Gly Gly Leu Gly Ser Gln Gly  
 165 170 175  
 Ala Gly Arg Gly Gly Gln Gly Ala Gly Ala Ala Ala Ala Ala Gly  
 180 185 190  
 Gly Ala Gly Gln Gly Gly Tyr Gly Gly Leu Gly Ser Gln Gly Ala Gly  
 195 200 205  
 Gln Gly Gly Tyr Gly Gly Leu Gly Ser Gln Gly Ala Gly Arg Gly Gly  
 210 215 220  
 Leu Gly Gly Gln Gly Ala Gly Ala Ala Ala Ala Ala Ala Gly Gly  
 225 230 235 240  
 Ala Gly Gln Gly Gly Leu Gly Ser Gln Gly Ala Gly Gln Gly Ala Gly  
 245 250 255  
 Ala Ala Ala Ala Ala Ala Gly Gly Ala Gly Gln Gly Gly Tyr Gly Gly  
 260 265 270  
 Leu Gly Ser Gln Gly Ala Gly Arg Gly Gly Gln Gly Ala Gly Ala Ala  
 275 280 285  
 Ala Ala Ala Ala Gly Gly Ala Gly Gln Gly Gly Tyr Gly Gly Leu Gly  
 290 295 300  
 Ser Gln Gly Ala Gly Gln Gly Gly Tyr Gly Gly Leu Gly Ser Gln Gly  
 305 310 315 320  
 Ala Gly Arg Gly Gly Leu Gly Gly Gln Gly Ala Gly Ala Ala Ala Ala  
 325 330 335  
 Ala Ala Ala Gly Gly Ala Gly Gln Gly Gly Leu Gly Ser Gln Gly Ala  
 340 345 350  
 Gly Gln Gly Ala Gly Ala Ala Ala Ala Ala Ala Gly Gly Ala Gly Gln  
 355 360 365  
 Gly Gly Tyr Gly Gly Leu Gly Ser Gln Gly Ala Gly Arg Gly Gly Gln  
 370 375 380  
 Gly Ala Gly Ala Ala Ala Ala Ala Ala Gly Gly Ala Gly Gln Gly Gly  
 385 390 395 400  
 Tyr Gly Gly Leu Gly Ser Gln Gly Ala Gly Gln Gly Gly Tyr Gly Gly  
 405 410 415  
 Leu Gly Ser Gln Gly Ala Gly Arg Gly Gly Leu Gly Gly Gln Gly Ala  
 420 425 430  
 Gly Ala Ala Ala Ala Ala Ala Ala Gly Gly Ala Gly Gln Gly Gly Leu  
 435 440 445  
 Gly Ser Gln Gly Ala Gly Gln Gly Ala Gly Ala Ala Ala Ala Ala Ala  
 450 455 460  
 Gly Gly Ala Gly Gln Gly Gly Tyr Gly Gly Leu Gly Ser Gln Gly Ala  
 465 470 475 480  
 Gly Arg Gly Gly Gln Gly Ala Gly Ala Ala Ala Ala Ala Gly Gly  
 485 490 495

Ala Gly Gln Gly Gly Tyr Gly Gly Leu Gly Ser Gln Gly Ala Gly Gln  
 500 505 510  
 Gly Gly Tyr Gly Gly Leu Gly Ser Gln Gly Ala Gly Arg Gly Gly Leu  
 515 520 525  
 Gly Gly Gln Gly Ala Gly Ala Ala Ala Ala Ala Gly Gly Ala  
 530 535 540  
 Gly Gln Gly Gly Leu Gly Ser Gln Gly Ala Gly Gln Gly Ala Gly Ala  
 545 550 555 560  
 Ala Ala Ala Ala Ala Gly Gly Ala Gly Gln Gly Gly Tyr Gly Gly Leu  
 565 570 575  
 Gly Ser Gln Gly Ala Gly Arg Gly Gly Gln Gly Ala Gly Ala Ala Ala  
 580 585 590  
 Ala Ala Ala Gly Gly Ala Gly Gln Gly Gly Tyr Gly Gly Leu Gly Ser  
 595 600 605  
 Gln Gly Ala Gly Gln Gly Gly Tyr Gly Gly Leu Gly Ser Gln Gly Ala  
 610 615 620  
 Gly Arg Gly Gly Leu Gly Gly Gln Gly Ala Gly Ala Ala Ala Ala Ala  
 625 630 635 640  
 Ala Ala Gly Gly Ala Gly Gln Gly Gly Leu Gly Ser Gln Gly Ala Gly  
 645 650 655  
 Gln Gly Ala Gly Ala Ala Ala Ala Ala Ala Gly Gly Ala Gly Gln Gly  
 660 665 670  
 Gly Tyr Gly Gly Leu Gly Ser Gln Gly Ala Gly Arg Gly Gly Gln Gly  
 675 680 685  
 Ala Gly Ala Ala Ala Ala Ala Ala Gly Gly Ala Gly Gln Gly Gly Tyr  
 690 695 700  
 Gly Gly Leu Gly Ser Gln Gly Ala Gly Gln Gly Gly Tyr Gly Gly Leu  
 705 710 715 720  
 Gly Ser Gln Gly Ala Gly Arg Gly Gly Leu Gly Gly Gln Gly Ala Gly  
 725 730 735  
 Ala Ala Ala Ala Ala Ala Ala Gly Gly Ala Gly Gln Gly Gly Leu Gly  
 740 745 750  
 Ser Gln Gly Ala Gly Gln Gly Ala Gly Ala Ala Ala Ala Ala Gly  
 755 760 765  
 Gly Ala Gly Gln Gly Gly Tyr Gly Gly Leu Gly Ser Gln Gly Ala Gly  
 770 775 780  
 Arg Gly Gly Gln Gly Ala Gly Ala Ala Ala Ala Ala Gly Gly Ala  
 785 790 795 800  
 Gly Gln Gly Gly Tyr Gly Gly Leu Gly  
 805

<210> 14  
 <211> 1617  
 <212> PRT  
 <213> Artificial Sequence

&lt;220&gt;

&lt;223&gt; Description of Artificial Sequence: Primer

&lt;400&gt; 14

Arg Ser Gln Gly Ala Gly Gln Gly Gly Tyr Gly Gly Leu Gly Ser Gln  
 1 5 10 15  
 Gly Ala Gly Arg Gly Gly Leu Gly Gly Gln Gly Ala Gly Ala Ala Ala  
 20 25 30  
 Ala Ala Ala Ala Gly Gly Ala Gly Gln Gly Gly Leu Gly Ser Gln Gly  
 35 40 45  
 Ala Gly Gln Gly Ala Gly Ala Ala Ala Ala Ala Gly Gly Ala Gly  
 50 55 60  
 Gln Gly Gly Tyr Gly Gly Leu Gly Ser Gln Gly Ala Gly Arg Gly Gly  
 65 70 75 80  
 Gln Gly Ala Gly Ala Ala Ala Ala Ala Gly Gly Ala Gly Gln Gly  
 85 90 95  
 Gly Tyr Gly Gly Leu Gly Ser Gln Gly Ala Gly Gln Gly Gly Tyr Gly  
 100 105 110  
 Gly Leu Gly Ser Gln Gly Ala Gly Arg Gly Gly Leu Gly Gly Gln Gly  
 115 120 125  
 Ala Gly Ala Ala Ala Ala Ala Ala Ala Gly Gly Ala Gly Gln Gly Gly  
 130 135 140  
 Leu Gly Ser Gln Gly Ala Gly Gln Gly Ala Gly Ala Ala Ala Ala Ala  
 145 150 155 160  
 Ala Gly Gly Ala Gly Gln Gly Gly Tyr Gly Gly Leu Gly Ser Gln Gly  
 165 170 175  
 Ala Gly Arg Gly Gly Gln Gly Ala Gly Ala Ala Ala Ala Ala Gly  
 180 185 190  
 Gly Ala Gly Gln Gly Gly Tyr Gly Gly Leu Gly Ser Gln Gly Ala Gly  
 195 200 205  
 Gln Gly Gly Tyr Gly Gly Leu Gly Ser Gln Gly Ala Gly Arg Gly Gly  
 210 215 220  
 Leu Gly Gly Gln Gly Ala Gly Ala Ala Ala Ala Ala Ala Gly Gly  
 225 230 235 240  
 Ala Gly Gln Gly Gly Leu Gly Ser Gln Gly Ala Gly Gln Gly Ala Gly  
 245 250 255  
 Ala Ala Ala Ala Ala Ala Gly Gly Ala Gly Gln Gly Gly Tyr Gly Gly  
 260 265 270  
 Leu Gly Ser Gln Gly Ala Gly Arg Gly Gly Gln Gly Ala Gly Ala Ala  
 275 280 285  
 Ala Ala Ala Ala Gly Gly Ala Gly Gln Gly Gly Tyr Gly Gly Leu Gly  
 290 295 300  
 Ser Gln Gly Ala Gly Gln Gly Gly Tyr Gly Gly Leu Gly Ser Gln Gly  
 305 310 315 320

Ala Gly Arg Gly Gly Leu Gly Gly Gln Gly Ala Gly Ala Ala Ala Ala  
 325 330 335  
 Ala Ala Ala Gly Gly Ala Gly Gln Gly Gly Leu Gly Ser Gln Gly Ala  
 340 345 350  
 Gly Gln Gly Ala Gly Ala Ala Ala Ala Ala Gly Gly Ala Gly Gln  
 355 360 365  
 Gly Gly Tyr Gly Gly Leu Gly Ser Gln Gly Ala Gly Arg Gly Gly Gln  
 370 375 380  
 Gly Ala Gly Ala Ala Ala Ala Ala Ala Gly Gly Ala Gly Gln Gly Gly  
 385 390 395 400  
 Tyr Gly Gly Leu Gly Ser Gln Gly Ala Gly Gln Gly Gly Tyr Gly Gly  
 405 410 415  
 Leu Gly Ser Gln Gly Ala Gly Arg Gly Gly Leu Gly Gly Gln Gly Ala  
 420 425 430  
 Gly Ala Ala Ala Ala Ala Ala Ala Gly Gly Ala Gly Gln Gly Gly Leu  
 435 440 445  
 Gly Ser Gln Gly Ala Gly Gln Gly Ala Gly Ala Ala Ala Ala Ala  
 450 455 460  
 Gly Gly Ala Gly Gln Gly Gly Tyr Gly Gly Leu Gly Ser Gln Gly Ala  
 465 470 475 480  
 Gly Arg Gly Gly Gln Gly Ala Gly Ala Ala Ala Ala Ala Gly Gly  
 485 490 495  
 Ala Gly Gln Gly Gly Tyr Gly Gly Leu Gly Ser Gln Gly Ala Gly Gln  
 500 505 510  
 Gly Gly Tyr Gly Gly Leu Gly Ser Gln Gly Ala Gly Arg Gly Gly Leu  
 515 520 525  
 Gly Gly Gln Gly Ala Gly Ala Ala Ala Ala Ala Ala Ala Gly Gly Ala  
 530 535 540  
 Gly Gln Gly Gly Leu Gly Ser Gln Gly Ala Gly Gln Gly Ala Gly Ala  
 545 550 555 560  
 Ala Ala Ala Ala Ala Gly Gly Ala Gly Gln Gly Gly Tyr Gly Gly Leu  
 565 570 575  
 Gly Ser Gln Gly Ala Gly Arg Gly Gly Gln Gly Ala Gly Ala Ala Ala  
 580 585 590  
 Ala Ala Ala Gly Gly Ala Gly Gln Gly Gly Tyr Gly Gly Leu Gly Ser  
 595 600 605  
 Gln Gly Ala Gly Gln Gly Gly Tyr Gly Gly Leu Gly Ser Gln Gly Ala  
 610 615 620  
 Gly Arg Gly Gly Leu Gly Gly Gln Gly Ala Gly Ala Ala Ala Ala Ala  
 625 630 635 640  
 Ala Ala Gly Gly Ala Gly Gln Gly Gly Leu Gly Ser Gln Gly Ala Gly  
 645 650 655



Gln Gly Ala Gly Ala Ala Ala Ala Ala Ala Gly Gly Ala Gly Gln Gly  
 660 665 670  
 Gly Tyr Gly Gly Leu Gly Ser Gln Gly Ala Gly Arg Gly Gly Gln Gly  
 675 680 685  
 Ala Gly Ala Ala Ala Ala Ala Ala Gly Gly Ala Gly Gln Gly Gly Tyr  
 690 695 700  
 Gly Gly Leu Gly Ser Gln Gly Ala Gly Gln Gly Gly Tyr Gly Gly Leu  
 705 710 715 720  
 Gly Ser Gln Gly Ala Gly Arg Gly Gly Leu Gly Gly Gln Gly Ala Gly  
 725 730 735  
 Ala Ala Ala Ala Ala Ala Ala Gly Gly Ala Gly Gln Gly Gly Leu Gly  
 740 745 750  
 Ser Gln Gly Ala Gly Gln Gly Ala Gly Ala Ala Ala Ala Ala Ala Gly  
 755 760 765  
 Gly Ala Gly Gln Gly Gly Tyr Gly Gly Leu Gly Ser Gln Gly Ala Gly  
 770 775 780  
 Arg Gly Gly Gln Gly Ala Gly Ala Ala Ala Ala Ala Ala Gly Gly Ala  
 785 790 795 800  
 Gly Gln Gly Gly Tyr Gly Gly Leu Gly Ser Gln Gly Ala Gly Gln Gly  
 805 810 815  
 Gly Tyr Gly Gly Leu Gly Ser Gln Gly Ala Gly Arg Gly Gly Leu Gly  
 820 825 830  
 Gly Gln Gly Ala Gly Ala Ala Ala Ala Ala Ala Gly Gly Ala Gly  
 835 840 845  
 Gln Gly Gly Leu Gly Ser Gln Gly Ala Gly Gln Gly Ala Gly Ala Ala  
 850 855 860  
 Ala Ala Ala Ala Gly Gly Ala Gly Gln Gly Gly Tyr Gly Gly Leu Gly  
 865 870 875 880  
 Ser Gln Gly Ala Gly Arg Gly Gly Gln Gly Ala Gly Ala Ala Ala Ala  
 885 890 895  
 Ala Ala Gly Gly Ala Gly Gln Gly Gly Tyr Gly Gly Leu Gly Ser Gln  
 900 905 910  
 Gly Ala Gly Gln Gly Gly Tyr Gly Gly Leu Gly Ser Gln Gly Ala Gly  
 915 920 925  
 Arg Gly Gly Leu Gly Gly Gln Gly Ala Gly Ala Ala Ala Ala Ala Ala  
 930 935 940  
 Ala Gly Gly Ala Gly Gln Gly Gly Leu Gly Ser Gln Gly Ala Gly Gln  
 945 950 955 960  
 Gly Ala Gly Ala Ala Ala Ala Ala Gly Gly Ala Gly Gln Gly Gly  
 965 970 975  
 Tyr Gly Gly Leu Gly Ser Gln Gly Ala Gly Arg Gly Gly Gln Gly Ala  
 980 985 990

Gly Ala Ala Ala Ala Ala Gly Gly Ala Gly Gln Gly Gly Tyr Gly  
995 1000 1005

Gly Leu Gly Ser Gln Gly Ala Gly Gln Gly Gly Tyr Gly Gly Leu Gly  
1010 1015 1020

Ser Gln Gly Ala Gly Arg Gly Gly Leu Gly Gly Gln Gly Ala Gly Ala  
1025 1030 1035 1040

Ala Ala Ala Ala Ala Ala Gly Gly Ala Gly Gln Gly Gly Leu Gly Ser  
1045 1050 1055

Gln Gly Ala Gly Gln Gly Ala Gly Ala Ala Ala Ala Ala Gly Gly  
1060 1065 1070

Ala Gly Gln Gly Gly Tyr Gly Gly Leu Gly Ser Gln Gly Ala Gly Arg  
1075 1080 1085

Gly Gly Gln Gly Ala Gly Ala Ala Ala Ala Ala Gly Gly Ala Gly  
1090 1095 1100

Gln Gly Gly Tyr Gly Gly Leu Gly Ser Gln Gly Ala Gly Gln Gly Gly  
1105 1110 1115 1120

Tyr Gly Gly Leu Gly Ser Gln Gly Ala Gly Arg Gly Gly Leu Gly Gly  
1125 1130 1135

Gln Gly Ala Gly Ala Ala Ala Ala Ala Gly Gly Ala Gly Gln  
1140 1145 1150

Gly Gly Leu Gly Ser Gln Gly Ala Gly Gln Gly Ala Gly Ala Ala Ala  
1155 1160 1165

Ala Ala Ala Gly Gly Ala Gly Gln Gly Gly Tyr Gly Gly Leu Gly Ser  
1170 1175 1180

Gln Gly Ala Gly Arg Gly Gly Gln Gly Ala Gly Ala Ala Ala Ala Ala  
1185 1190 1195 1200

Ala Gly Gly Ala Gly Gln Gly Gly Tyr Gly Gly Leu Gly Ser Gln Gly  
1205 1210 1215

Ala Gly Gln Gly Gly Tyr Gly Gly Leu Gly Ser Gln Gly Ala Gly Arg  
1220 1225 1230

Gly Gly Leu Gly Gly Gln Gly Ala Gly Ala Ala Ala Ala Ala Ala Ala  
1235 1240 1245

Gly Gly Ala Gly Gln Gly Gly Leu Gly Ser Gln Gly Ala Gly Gln Gly  
1250 1255 1260

Ala Gly Ala Ala Ala Ala Ala Gly Gly Ala Gly Gln Gly Gly Tyr  
1265 1270 1275 1280

Gly Gly Leu Gly Ser Gln Gly Ala Gly Arg Gly Gly Gln Gly Ala Gly  
1285 1290 1295

Ala Ala Ala Ala Ala Ala Gly Gly Ala Gly Gln Gly Gly Tyr Gly Gly  
1300 1305 1310

Leu Gly Ser Gln Gly Ala Gly Gln Gly Gly Tyr Gly Gly Leu Gly Ser  
1315 1320 1325

Gln Gly Ala Gly Arg Gly Gly Leu Gly Gly Gln Gly Ala Gly Ala Ala  
 1330 1335 1340  
 Ala Ala Ala Ala Ala Gly Gly Ala Gly Gln Gly Gly Leu Gly Ser Gln  
 1345 1350 1355 1360  
 Gly Ala Gly Gln Gly Ala Gly Ala Ala Ala Ala Ala Gly Gly Ala  
 1365 1370 1375  
 Gly Gln Gly Gly Tyr Gly Gly Leu Gly Ser Gln Gly Ala Gly Arg Gly  
 1380 1385 1390  
 Gly Gln Gly Ala Gly Ala Ala Ala Ala Ala Gly Gly Ala Gly Gln  
 1395 1400 1405  
 Gly Gly Tyr Gly Gly Leu Gly Ser Gln Gly Ala Gly Gln Gly Gly Tyr  
 1410 1415 1420  
 Gly Gly Leu Gly Ser Gln Gly Ala Gly Arg Gly Gly Leu Gly Gly Gln  
 1425 1430 1435 1440  
 Gly Ala Gly Ala Ala Ala Ala Ala Ala Gly Gly Ala Gly Gln Gly  
 1445 1450 1455  
 Gly Leu Gly Ser Gln Gly Ala Gly Gln Gly Ala Gly Ala Ala Ala Ala  
 1460 1465 1470  
 Ala Ala Gly Gly Ala Gly Gln Gly Gly Tyr Gly Gly Leu Gly Ser Gln  
 1475 1480 1485  
 Gly Ala Gly Arg Gly Gly Gln Gly Ala Gly Ala Ala Ala Ala Ala  
 1490 1495 1500  
 Gly Gly Ala Gly Gln Gly Gly Tyr Gly Gly Leu Gly Ser Gln Gly Ala  
 1505 1510 1515 1520  
 Gly Gln Gly Gly Tyr Gly Gly Leu Gly Ser Gln Gly Ala Gly Arg Gly  
 1525 1530 1535  
 Gly Leu Gly Gly Gln Gly Ala Gly Ala Ala Ala Ala Ala Ala Gly  
 1540 1545 1550  
 Gly Ala Gly Gln Gly Gly Leu Gly Ser Gln Gly Ala Gly Gln Gly Ala  
 1555 1560 1565  
 Gly Ala Ala Ala Ala Ala Ala Gly Gly Ala Gly Gln Gly Gly Tyr Gly  
 1570 1575 1580  
 Gly Leu Gly Ser Gln Gly Ala Gly Arg Gly Gly Gln Gly Ala Gly Ala  
 1585 1590 1595 1600  
 Ala Ala Ala Ala Ala Gly Gly Ala Gly Gln Gly Gly Tyr Gly Gly Leu  
 1605 1610 1615

Gly

<210> 15  
 <211> 50  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> Description of Artificial Sequence: Primer

<400> 15  
 gatctccatg gctagatcta gaggatccca tcaccatcac catcactaag 50  
  
 <210> 16  
 <211> 50  
 <212> DNA  
 <213> Artificial Sequence  
  
 <220>  
 <223> Description of Artificial Sequence: Primer  
  
 <400> 16  
 aattcttagt gatggtgatg gtgatgggat cctctagatc tagccatgga 50  
  
 <210> 17  
 <211> 6  
 <212> PRT  
 <213> Artificial Sequence  
  
 <220>  
 <223> Description of Artificial Sequence: SPL repeat  
  
 <400> 17  
 Ala Arg Ser Arg Gly Ser  
 1 5  
  
 <210> 18  
 <211> 50  
 <212> DNA  
 <213> Artificial Sequence  
  
 <220>  
 <223> Description of Artificial Sequence: Adapter sequence  
  
 <400> 18  
 gatctccatg gctagatcta gaggatccca tcaccatcac catcactaag 50  
  
 <210> 19  
 <211> 50  
 <212> DNA  
 <213> Artificial Sequence  
  
 <220>  
 <223> Description of Artificial Sequence: Adapter sequence  
  
 <400> 19  
 aggtaccgat ctagatctcc tagggtagtg gtagtggtag tgattcttaa 50  
  
 <210> 20  
 <211> 13  
 <212> PRT  
 <213> Artificial Sequence  
  
 <220>  
 <223> Description of Artificial Sequence: Adapter peptide  
  
 <400> 20  
 Met Ala Arg Ser Arg Gly Ser His His His His His His  
 1 5 10  
  
 <210> 21  
 <211> 2457

<212> DNA  
 <213> Artificial Sequence

<220>  
 <223> Description of Artificial Sequence: DP-1B 8mer coding region  
 with His tab

<400> 21  
 atggctagat ctcaaggagc cgggtcaagggt gggttacggag gtctgggatac tcaagggtgct 60  
 ggacgtgggtg gtcttggtgg tcagggtgcc gggtgccgccg ctgccgccgc cgctgggtgg 120  
 gctggacaag gtgggtttggg atctcaggga gctgggtcaag gtgccgggtgc tgctgccgct 180  
 gctgccggag gtgccgggtca ggggtggatac gggtggacttg gatctcaggg tgctggtaga 240  
 ggtggacaag gtgccggagc tgccgctgcc gctgccgggtg gtgctgggtca aggaggttac 300  
 ggtgggtcttg gatctcaagg agccgggtcaa ggtgggttacg gaggtctggg atctcaagggt 360  
 gctggacgtg gtgggtcttg tggtcagggt gccgggtgccg ccgctgccgc cgccgctggt 420  
 ggtgctggac aagggtgggtt gggatctcag ggagctggtc aagggtgccg tgctgctgcc 480  
 gctgctgccg gaggtgccgg tcagggtgga tacgggtggac ttggatctca ggggtgctggt 540  
 agaggtggac aagggtgccg agctgccgct gccgctgccg gtgggtgctgg tcaaggaggt 600  
 tacgggtggc ttggatctca aggagccgggt caagggtggt acggaggtct gggatctcaa 660  
 ggtgctggac gtgggtggtc ttgggtggtcagg gggtgccgggt ccgccgctgc cgccgccgct 720  
 ggtgggtgctg gacaagggtg tttgggatct caggagctg gtcaagggtgc cggtgctgct 780  
 gccgctgctg ccggaggtgc cgggtcagggt ggatacgggt gacttggtac tcagggtgct 840  
 ggtagaggtg gacaagggtgc cggagctgcc gctgccgctg ccgggtgggtc tgggtcaagga 900  
 gggttacgggtg gtcttggtac tcaaggagcc ggtcaagggtg gttacggagg tctgggatct 960  
 caagggtgctg gacgtgggtg tcttggtggt cagggtgccg gtgccgccgc tgccgccgcc 1020  
 gctgggtgggtg ctggacaagg tgggtttggga tctcagggtg ctgggtcaagg tgccgggtgct 1080  
 gctgccgctg ctgccggagg tgccgggtcag gggtggatac gtggacttggt atctcagggt 1140  
 gctggtagag gtggacaagg tgccggagct gccgctgccg ctgccgggtg tgctgggtcaa 1200  
 ggaggttacg gtgggtcttg atctcaagga gccgggtcaag gtgggttacg aggtctggga 1260  
 tctcaagggtg ctggacgtgg tgggtcttggt ggtcagggtg ccgggtgccgc cgctgccgcc 1320  
 gccgctggtg gtgctggaca aggtgggtttg ggatctcagg gagctgggtca aggtgccggt 1380  
 gctgctgccg ctgctgccgg aggtgccgggt cagggtggat acgggtggact tggatctcag 1440  
 ggtgctggta gaggtggaca aggtgccgga gctgccgctg ccgctgccgc tgggtgctggt 1500  
 caaggaggtt acgggtggtc tggatctcaa ggagccgggtc aagggtggtta cggaggtctg 1560  
 ggatctcaag gtgctggacg tgggtggtctt ggtggtcagg gtgccgggtgc cgccgctgcc 1620  
 gccgccgctg gtgggtgctg acaagggtggt ttgggatctc agggagctgg tcaagggtgcc 1680  
 ggtgctgctg ccgctgctgc cggaggtgcc gggtcagggtg gatacgggtg acttggtatct 1740  
 cagggtgctg gtagaggtgg acaagggtgcc ggagctgccg ctgccgctgc cggtgggtgct 1800  
 ggtcaaggag gttacgggtg tcttggtatct caaggagccg gtcaagggtg ttacggaggt 1860  
 ctgggatctc aagggtgctg acgtgggtggt cttgggtggtc aggggtgccg tgccgccgct 1920  
 gccgccgccg ctgggtggtg tggacaagggt ggtttgggat ctcagggagc tgggtcaagg 1980  
 gccggtgctg ctgccgctgc tgccggaggt gccggtcagg gtggatacgg tggacttgga 2040  
 tctcagggtg ctggttagag tggacaagggt gccggagctg ccgctgccgc tgccgggtggt 2100  
 gctgggtcaag gaggttacgg tgggtcttgga tctcaaggag ccgggtcaagg tgggttacgga 2160  
 ggtctgggat ctcaagggtgc tggacgtggt ggtcttggtg gtcagggtgc cggtgccgcc 2220  
 gctgccgccg ccgctggtgg tgctggacaa ggtgggtttg gatctcaggg agctgggtcaa 2280  
 ggtgccgggt ctgctgccgc tgctgccgga ggtgccgggtc aggggtggata cggtggactt 2340  
 ggatctcagg gtgctggtag aggtggacaa ggtgccggag ctgccgctgc cgctgccgggt 2400  
 ggtgctgggtc aaggaggtta cgggtggtctt ggatcccatc accatcacca tcactaa 2457

<210> 22  
 <211> 818  
 <212> PRT  
 <213> Artificial Sequence

<220>  
 <223> Description of Artificial Sequence: DP-1B 8mer with His Tag

<400> 22  
 Met Ala Arg Ser Gln Gly Ala Gly Gln Gly Gly Tyr Gly Gly Leu Gly  
 1 5 10 15  
 Ser Gln Gly Ala Gly Arg Gly Gly Leu Gly Gly Gln Gly Ala Gly Ala  
 20 25 30



Ala Ala Ala Ala Ala Ala Gly Gly Ala Gly Gln Gly Gly Leu Gly Ser  
 35 40 45  
 Gln Gly Ala Gly Gln Gly Ala Gly Ala Ala Ala Ala Ala Gly Gly  
 50 55 60  
 Ala Gly Gln Gly Gly Tyr Gly Gly Leu Gly Ser Gln Gly Ala Gly Arg  
 65 70 75 80  
 Gly Gly Gln Gly Ala Gly Ala Ala Ala Ala Ala Gly Gly Ala Gly  
 85 90 95  
 Gln Gly Gly Tyr Gly Gly Leu Gly Ser Gln Gly Ala Gly Gln Gly Gly  
 100 105 110  
 Tyr Gly Gly Leu Gly Ser Gln Gly Ala Gly Arg Gly Gly Leu Gly Gly  
 115 120 125  
 Gln Gly Ala Gly Ala Ala Ala Ala Ala Ala Gly Gly Ala Gly Gln  
 130 135 140  
 Gly Gly Leu Gly Ser Gln Gly Ala Gly Gln Gly Ala Gly Ala Ala Ala  
 145 150 155 160  
 Ala Ala Ala Gly Gly Ala Gly Gln Gly Gly Tyr Gly Gly Leu Gly Ser  
 165 170 175  
 Gln Gly Ala Gly Arg Gly Gly Gln Gly Ala Gly Ala Ala Ala Ala Ala  
 180 185 190  
 Ala Gly Gly Ala Gly Gln Gly Gly Tyr Gly Gly Leu Gly Ser Gln Gly  
 195 200 205  
 Ala Gly Gln Gly Gly Tyr Gly Gly Leu Gly Ser Gln Gly Ala Gly Arg  
 210 215 220  
 Gly Gly Leu Gly Gly Gln Gly Ala Gly Ala Ala Ala Ala Ala Ala Ala  
 225 230 235 240  
 Gly Gly Ala Gly Gln Gly Gly Leu Gly Ser Gln Gly Ala Gly Gln Gly  
 245 250 255  
 Ala Gly Ala Ala Ala Ala Ala Ala Gly Gly Ala Gly Gln Gly Gly Tyr  
 260 265 270  
 Gly Gly Leu Gly Ser Gln Gly Ala Gly Arg Gly Gly Gln Gly Ala Gly  
 275 280 285  
 Ala Ala Ala Ala Ala Ala Gly Gly Ala Gly Gln Gly Gly Tyr Gly Gly  
 290 295 300  
 Leu Gly Ser Gln Gly Ala Gly Gln Gly Gly Tyr Gly Gly Leu Gly Ser  
 305 310 315 320  
 Gln Gly Ala Gly Arg Gly Gly Leu Gly Gly Gln Gly Ala Gly Ala Ala  
 325 330 335  
 Ala Ala Ala Ala Ala Gly Gly Ala Gly Gln Gly Gly Leu Gly Ser Gln  
 340 345 350  
 Gly Ala Gly Gln Gly Ala Gly Ala Ala Ala Ala Ala Ala Gly Gly Ala  
 355 360 365

Gly Gln Gly Gly Tyr Gly Gly Leu Gly Ser Gln Gly Ala Gly Arg Gly  
 370 375 380  
 Gly Gln Gly Ala Gly Ala Ala Ala Ala Ala Gly Gly Ala Gly Gln  
 385 390 395 400  
 Gly Gly Tyr Gly Gly Leu Gly Ser Gln Gly Ala Gly Gln Gly Gly Tyr  
 405 410 415  
 Gly Gly Leu Gly Ser Gln Gly Ala Gly Arg Gly Gly Leu Gly Gly Gln  
 420 425 430  
 Gly Ala Gly Ala Ala Ala Ala Ala Ala Gly Gly Ala Gly Gln Gly  
 435 440 445  
 Gly Leu Gly Ser Gln Gly Ala Gly Gln Gly Ala Gly Ala Ala Ala Ala  
 450 455 460  
 Ala Ala Gly Gly Ala Gly Gln Gly Gly Tyr Gly Gly Leu Gly Ser Gln  
 465 470 475 480  
 Gly Ala Gly Arg Gly Gly Gln Gly Ala Gly Ala Ala Ala Ala Ala Ala  
 485 490 495  
 Gly Gly Ala Gly Gln Gly Gly Tyr Gly Gly Leu Gly Ser Gln Gly Ala  
 500 505 510  
 Gly Gln Gly Gly Tyr Gly Gly Leu Gly Ser Gln Gly Ala Gly Arg Gly  
 515 520 525  
 Gly Leu Gly Gly Gln Gly Ala Gly Ala Ala Ala Ala Ala Ala Gly  
 530 535 540  
 Gly Ala Gly Gln Gly Gly Leu Gly Ser Gln Gly Ala Gly Gln Gly Ala  
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 Gly Ala Ala Ala Ala Ala Ala Gly Gly Ala Gly Gln Gly Gly Tyr Gly  
 565 570 575  
 Gly Leu Gly Ser Gln Gly Ala Gly Arg Gly Gly Gln Gly Ala Gly Ala  
 580 585 590  
 Ala Ala Ala Ala Ala Gly Gly Ala Gly Gln Gly Gly Tyr Gly Gly Leu  
 595 600 605  
 Gly Ser Gln Gly Ala Gly Gln Gly Gly Tyr Gly Gly Leu Gly Ser Gln  
 610 615 620  
 Gly Ala Gly Arg Gly Gly Leu Gly Gly Gln Gly Ala Gly Ala Ala Ala  
 625 630 635 640  
 Ala Ala Ala Ala Gly Gly Ala Gly Gln Gly Gly Leu Gly Ser Gln Gly  
 645 650 655  
 Ala Gly Gln Gly Ala Gly Ala Ala Ala Ala Ala Gly Gly Ala Gly  
 660 665 670  
 Gln Gly Gly Tyr Gly Gly Leu Gly Ser Gln Gly Ala Gly Arg Gly Gly  
 675 680 685  
 Gln Gly Ala Gly Ala Ala Ala Ala Ala Gly Gly Ala Gly Gln Gly  
 690 695 700

Gly Tyr Gly Gly Leu Gly Ser Gln Gly Ala Gly Gln Gly Gly Tyr Gly  
705 710 715 720

Gly Leu Gly Ser Gln Gly Ala Gly Arg Gly Gly Leu Gly Gly Gln Gly  
725 730 735

Ala Gly Ala Ala Ala Ala Ala Ala Ala Gly Gly Ala Gly Gln Gly Gly  
740 745 750

Leu Gly Ser Gln Gly Ala Gly Gln Gly Ala Gly Ala Ala Ala Ala Ala  
755 760 765

Ala Gly Gly Ala Gly Gln Gly Gly Tyr Gly Gly Leu Gly Ser Gln Gly  
770 775 780

Ala Gly Arg Gly Gly Gln Gly Ala Gly Ala Ala Ala Ala Ala Gly  
785 790 795 800

Gly Ala Gly Gln Gly Gly Tyr Gly Gly Leu Gly Ser His His His His  
805 810 815

His His

<210> 23  
<211> 4881  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: DP-1B 16 mere coding region  
with His Tag

<400> 23  
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gctggacaag gtggttttggg atctcaggga gctgggtcaag gtgccgggtgc tgctgccgct 180  
gctgccggag gtgccgggtca ggggtggatac ggtggacttg gatctcaggg tgctggtaga 240  
gggtggacaag gtgccgggagc tgccgctgcc gctgccgggtg gtgctgggtca aggaggttac 300  
gggtggtcttg gatctcaagg agccgggtcaa ggtgggttacg gaggtctggg atctcaagggt 360  
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gctgctgccg gaggtgccgg tcagggtgga tacgggtggac ttggatctca ggggtgctggt 540  
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gggtggtgctg gacaagggtg tttgggatct caggagctg gtcaagggtgc cggtgctgct 780  
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gctggtggtg ctggacaagg tggttttggga tctcagggtg ctggtcaagg tgccggtgct 1080  
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catcaccatc accatcacta a 4881

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&lt;210&gt; 24

&lt;211&gt; 1626

&lt;212&gt; PRT

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Description of Artificial Sequence: DP-1B 16mer with His Tag

&lt;400&gt; 24

Met Ala Arg Ser Gln Gly Ala Gly Gln Gly Gly Tyr Gly Gly Leu Gly  
1 5 10 15



Ser Gln Gly Ala Gly Arg Gly Gly Leu Gly Gly Gln Gly Ala Gly Ala  
 20 25 30  
 Ala Ala Ala Ala Ala Ala Gly Gly Ala Gly Gln Gly Gly Leu Gly Ser  
 35 40 45  
 Gln Gly Ala Gly Gln Gly Ala Gly Ala Ala Ala Ala Ala Ala Gly Gly  
 50 55 60  
 Ala Gly Gln Gly Gly Tyr Gly Gly Leu Gly Ser Gln Gly Ala Gly Arg  
 65 70 75 80  
 Gly Gly Gln Gly Ala Gly Ala Ala Ala Ala Ala Gly Gly Ala Gly  
 85 90 95  
 Gln Gly Gly Tyr Gly Gly Leu Gly Ser Gln Gly Ala Gly Gln Gly Gly  
 100 105 110  
 Tyr Gly Gly Leu Gly Ser Gln Gly Ala Gly Arg Gly Gly Leu Gly Gly  
 115 120 125  
 Gln Gly Ala Gly Ala Ala Ala Ala Ala Ala Gly Gly Ala Gly Gln  
 130 135 140  
 Gly Gly Leu Gly Ser Gln Gly Ala Gly Gln Gly Ala Gly Ala Ala Ala  
 145 150 155 160  
 Ala Ala Ala Gly Gly Ala Gly Gln Gly Gly Tyr Gly Gly Leu Gly Ser  
 165 170 175  
 Gln Gly Ala Gly Arg Gly Gly Gln Gly Ala Gly Ala Ala Ala Ala Ala  
 180 185 190  
 Ala Gly Gly Ala Gly Gln Gly Gly Tyr Gly Gly Leu Gly Ser Gln Gly  
 195 200 205  
 Ala Gly Gln Gly Gly Tyr Gly Gly Leu Gly Ser Gln Gly Ala Gly Arg  
 210 215 220  
 Gly Gly Leu Gly Gly Gln Gly Gly Ala Gly Ala Ala Ala Ala Ala Ala  
 225 230 235 240  
 Gly Gly Ala Gly Gln Gly Gly Leu Gly Ser Gln Gly Ala Gly Gln Gly  
 245 250 255  
 Ala Gly Ala Ala Ala Ala Ala Ala Gly Gly Ala Gly Gln Gly Gly Tyr  
 260 265 270  
 Gly Gly Leu Gly Ser Gln Gly Ala Gly Arg Gly Gly Gln Gly Ala Gly  
 275 280 285  
 Ala Ala Ala Ala Ala Ala Gly Gly Ala Gly Gln Gly Gly Tyr Gly Gly  
 290 295 300  
 Leu Gly Ser Gln Gly Ala Gly Gln Gly Gly Tyr Gly Gly Leu Gly Ser  
 305 310 315 320  
 Gln Gly Ala Gly Arg Gly Gly Leu Gly Gly Gln Gly Ala Gly Ala Ala  
 325 330 335  
 Ala Ala Ala Ala Ala Gly Gly Ala Gly Gln Gly Gly Leu Gly Ser Gln  
 340 345 350



Gly Ala Gly Gln Gly Ala Gly Ala Ala Ala Ala Ala Gly Gly Ala  
 355 360 365  
 Gly Gln Gly Gly Tyr Gly Gly Leu Gly Ser Gln Gly Ala Gly Arg Gly  
 370 375 380  
 Gly Gln Gly Ala Gly Ala Ala Ala Ala Ala Gly Gly Ala Gly Gln  
 385 390 395 400  
 Gly Gly Tyr Gly Gly Leu Gly Ser Gln Gly Ala Gly Gln Gly Gly Tyr  
 405 410 415  
 Gly Gly Leu Gly Ser Gln Gly Ala Gly Arg Gly Gly Leu Gly Gly Gln  
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 Gly Ala Gly Ala Ala Ala Ala Ala Ala Ala Gly Gly Ala Gly Gln Gly  
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 Gly Leu Gly Ser Gln Gly Ala Gly Gln Gly Ala Gly Ala Ala Ala Ala  
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 Ala Ala Gly Gly Ala Gly Gln Gly Gly Tyr Gly Gly Leu Gly Ser Gln  
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 485 490 495  
 Gly Gly Ala Gly Gln Gly Gly Tyr Gly Gly Leu Gly Ser Gln Gly Ala  
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 Gly Gln Gly Gly Tyr Gly Gly Leu Gly Ser Gln Gly Ala Gly Arg Gly  
 515 520 525  
 Gly Leu Gly Gly Gln Gly Ala Gly Ala Ala Ala Ala Ala Ala Ala Gly  
 530 535 540  
 Gly Ala Gly Gln Gly Gly Leu Gly Ser Gln Gly Ala Gly Gln Gly Ala  
 545 550 555 560  
 Gly Ala Ala Ala Ala Ala Ala Gly Gly Ala Gly Gln Gly Gly Tyr Gly  
 565 570 575  
 Gly Leu Gly Ser Gln Gly Ala Gly Arg Gly Gly Gln Gly Ala Gly Ala  
 580 585 590  
 Ala Ala Ala Ala Ala Gly Gly Ala Gly Gln Gly Gly Tyr Gly Gly Leu  
 595 600 605  
 Gly Ser Gln Gly Ala Gly Gln Gly Gly Tyr Gly Gly Leu Gly Ser Gln  
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 625 630 635 640  
 Ala Ala Ala Ala Gly Gly Ala Gly Gln Gly Gly Leu Gly Ser Gln Gly  
 645 650 655  
 Ala Gly Gln Gly Ala Gly Ala Ala Ala Ala Ala Gly Gly Ala Gly  
 660 665 670  
 Gln Gly Gly Tyr Gly Gly Leu Gly Ser Gln Gly Ala Gly Arg Gly Gly  
 675 680 685

Gln Gly Ala Gly Ala Ala Ala Ala Ala Ala Gly Gly Ala Gly Gln Gly  
 690 695 700  
 Gly Tyr Gly Gly Leu Gly Ser Gln Gly Ala Gly Gln Gly Gly Tyr Gly  
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 Gly Leu Gly Ser Gln Gly Ala Gly Arg Gly Gly Leu Gly Gly Gln Gly  
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 Ala Gly Ala Ala Ala Ala Ala Ala Ala Gly Gly Ala Gly Gln Gly Gly  
 740 745 750  
 Leu Gly Ser Gln Gly Ala Gly Gln Gly Ala Gly Ala Ala Ala Ala  
 755 760 765  
 Ala Gly Gly Ala Gly Gln Gly Gly Tyr Gly Gly Leu Gly Ser Gln Gly  
 770 775 780  
 Ala Gly Arg Gly Gly Gln Gly Ala Gly Ala Ala Ala Ala Ala Gly  
 785 790 795 800  
 Gly Ala Gly Gln Gly Gly Tyr Gly Gly Leu Gly Ser Gln Gly Ala Gly  
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 820 825 830  
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 Ala Gly Gln Gly Gly Leu Gly Ser Gln Gly Ala Gly Gln Gly Ala Gly  
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 865 870 875 880  
 Leu Gly Ser Gln Gly Ala Gly Arg Gly Gly Gln Gly Ala Gly Ala Ala  
 885 890 895  
 Ala Ala Ala Ala Gly Gly Ala Gly Gln Gly Gly Tyr Gly Gly Leu Gly  
 900 905 910  
 Ser Gln Gly Ala Gly Gln Gly Gly Tyr Gly Gly Leu Gly Ser Gln Gly  
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 Ala Gly Arg Gly Gly Leu Gly Gly Gln Gly Ala Gly Ala Ala Ala Ala  
 930 935 940  
 Ala Ala Ala Gly Gly Ala Gly Gln Gly Gly Leu Gly Ser Gln Gly Ala  
 945 950 955 960  
 Gly Gln Gly Ala Gly Ala Ala Ala Ala Ala Ala Gly Gly Ala Gly Gln  
 965 970 975  
 Gly Gly Tyr Gly Gly Leu Gly Ser Gln Gly Ala Gly Arg Gly Gly Gln  
 980 985 990  
 Gly Ala Gly Ala Ala Ala Ala Ala Gly Gly Ala Gly Gln Gly Gly  
 995 1000 1005  
 Tyr Gly Gly Leu Gly Ser Gln Gly Ala Gly Gln Gly Gly Tyr Gly Gly  
 1010 1015 1020

Leu Gly Ser Gln Gly Ala Gly Arg Gly Gly Leu Gly Gly Gln Gly Ala  
 1025 1030 1035 1040  
 Gly Ala Ala Ala Ala Ala Ala Ala Gly Gly Ala Gly Gln Gly Gly Leu  
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 Gly Ser Gln Gly Ala Gly Gln Gly Ala Gly Ala Ala Ala Ala Ala  
 1060 1065 1070  
 Gly Gly Ala Gly Gln Gly Gly Tyr Gly Gly Leu Gly Ser Gln Gly Ala  
 1075 1080 1085  
 Gly Arg Gly Gly Gln Gly Ala Gly Ala Ala Ala Ala Ala Ala Gly Gly  
 1090 1095 1100  
 Ala Gly Gln Gly Gly Tyr Gly Gly Leu Gly Ser Gln Gly Ala Gly Gln  
 1105 1110 1115 1120  
 Gly Gly Tyr Gly Gly Leu Gly Ser Gln Gly Ala Gly Arg Gly Gly Leu  
 1125 1130 1135  
 Gly Gly Gln Gly Ala Gly Ala Ala Ala Ala Ala Ala Gly Gly Ala  
 1140 1145 1150  
 Gly Gln Gly Gly Leu Gly Ser Gln Gly Ala Gly Gln Gly Ala Gly Ala  
 1155 1160 1165  
 Ala Ala Ala Ala Ala Gly Gly Ala Gly Gln Gly Gly Tyr Gly Gly Leu  
 1170 1175 1180  
 Gly Ser Gln Gly Ala Gly Arg Gly Gly Gln Gly Ala Gly Ala Ala Ala  
 1185 1190 1195 1200  
 Ala Ala Ala Gly Gly Ala Gly Gln Gly Gly Tyr Gly Gly Leu Gly Ser  
 1205 1210 1215  
 Gln Gly Ala Gly Gln Gly Gly Tyr Gly Gly Leu Gly Ser Gln Gly Ala  
 1220 1225 1230  
 Gly Arg Gly Gly Leu Gly Gly Gln Gly Ala Gly Ala Ala Ala Ala Ala  
 1235 1240 1245  
 Ala Ala Gly Gly Ala Gly Gln Gly Gly Leu Gly Ser Gln Gly Ala Gly  
 1250 1255 1260  
 Gln Gly Ala Gly Ala Ala Ala Ala Ala Gly Gly Ala Gly Gln Gly  
 1265 1270 1275 1280  
 Gly Tyr Gly Gly Leu Gly Ser Gln Gly Ala Gly Arg Gly Gly Gln Gly  
 1285 1290 1295  
 Ala Gly Ala Ala Ala Ala Ala Gly Gly Ala Gly Gln Gly Gly Tyr  
 1300 1305 1310  
 Gly Gly Leu Gly Ser Gln Gly Ala Gly Gln Gly Gly Tyr Gly Gly Leu  
 1315 1320 1325  
 Gly Ser Gln Gly Ala Gly Arg Gly Gly Leu Gly Gly Gln Gly Ala Gly  
 1330 1335 1340  
 Ala Ala Ala Ala Ala Ala Ala Gly Gly Ala Gly Gln Gly Gly Leu Gly  
 1345 1350 1355 1360

Ser Gln Gly Ala Gly Gln Gly Ala Gly Ala Ala Ala Ala Ala Gly  
 1365 1370 1375

Gly Ala Gly Gln Gly Gly Tyr Gly Gly Leu Gly Ser Gln Gly Ala Gly  
 1380 1385 1390

Arg Gly Gly Gln Gly Ala Gly Ala Ala Ala Ala Ala Gly Gly Ala  
 1395 1400 1405

Gly Gln Gly Gly Tyr Gly Gly Leu Gly Ser Gln Gly Ala Gly Gln Gly  
 1410 1415 1420

Gly Tyr Gly Gly Leu Gly Ser Gln Gly Ala Gly Arg Gly Gly Leu Gly  
 1425 1430 1435 1440

Gly Gln Gly Ala Gly Ala Ala Ala Ala Ala Ala Gly Gly Ala Gly  
 1445 1450 1455

Gln Gly Gly Leu Gly Ser Gln Gly Ala Gly Gln Gly Ala Gly Ala Ala  
 1460 1465 1470

Ala Ala Ala Ala Gly Gly Ala Gly Gln Gly Gly Tyr Gly Gly Leu Gly  
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Ser Gln Gly Ala Gly Arg Gly Gly Gln Gly Ala Gly Ala Ala Ala Ala  
 1490 1495 1500

Ala Ala Gly Gly Ala Gly Gln Gly Gly Tyr Gly Gly Leu Gly Ser Gln  
 1505 1510 1515 1520

Gly Ala Gly Gln Gly Gly Tyr Gly Gly Leu Gly Ser Gln Gly Ala Gly  
 1525 1530 1535

Arg Gly Gly Leu Gly Gly Gln Gly Ala Gly Ala Ala Ala Ala Ala Ala  
 1540 1545 1550

Ala Gly Gly Ala Gly Gln Gly Gly Leu Gly Ser Gln Gly Ala Gly Gln  
 1555 1560 1565

Gly Ala Gly Ala Ala Ala Ala Ala Ala Gly Gly Ala Gly Gln Gly Gly  
 1570 1575 1580

Tyr Gly Gly Leu Gly Ser Gln Gly Ala Gly Arg Gly Gly Gln Gly Ala  
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Gly Ala Ala Ala Ala Ala Ala Gly Gly Ala Gly Gln Gly Gly Tyr Gly  
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Gly Leu Gly Ser His His His His His His  
 1620 1625

<210> 25

<211> 19

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: DP-1B immunogenic region

<400> 25

Cys Gly Ala Gly Gln Gly Gly Tyr Gly Gly Leu Gly Ser Gly Gly Ala  
 1 5 10 15

Gly Arg Gly

<210> 26  
<211> 20  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Primer

<400> 26  
gctcgacgtt gtcactgaag 20

<210> 27  
<211> 20  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Primer

<400> 27  
tcgtccagat catcctgac 20

<210> 28  
<211> 20  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Primer

<400> 28  
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<210> 29  
<211> 21  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Primer

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tagccatggt tagtatatct t 21



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- (72) Inventor: YANG, Jianjun, Gene: 7 Kent Drive, Hockessin, DE 19707 (US).  
*For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*



**WO 01/90389 A3**

(54) Title: PRODUCTION OF SILK-LIKE PROTEINS IN PLANTS

(57) Abstract: The invention provides methods for the production of silks and silk-like proteins (SLP's) in green plants. Expression of SLP's has been achieved in both seed and leaf tissue in green plants.

# INTERNATIONAL SEARCH REPORT

International Application No.  
PCT/US 01/16937

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N15/82 C07K14/435 C12P21/02 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N C07K C12P A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, BIOSIS, MEDLINE, SEQUENCE SEARCH, CHEM ABS Data

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 94 29450 A (DU PONT ;FAHNESTOCK STEPHEN R (US)) 22 December 1994 (1994-12-22) cited in the application the whole document	1,5-10, 12,14, 16-20
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☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

2 April 2002

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# INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 01/16937

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